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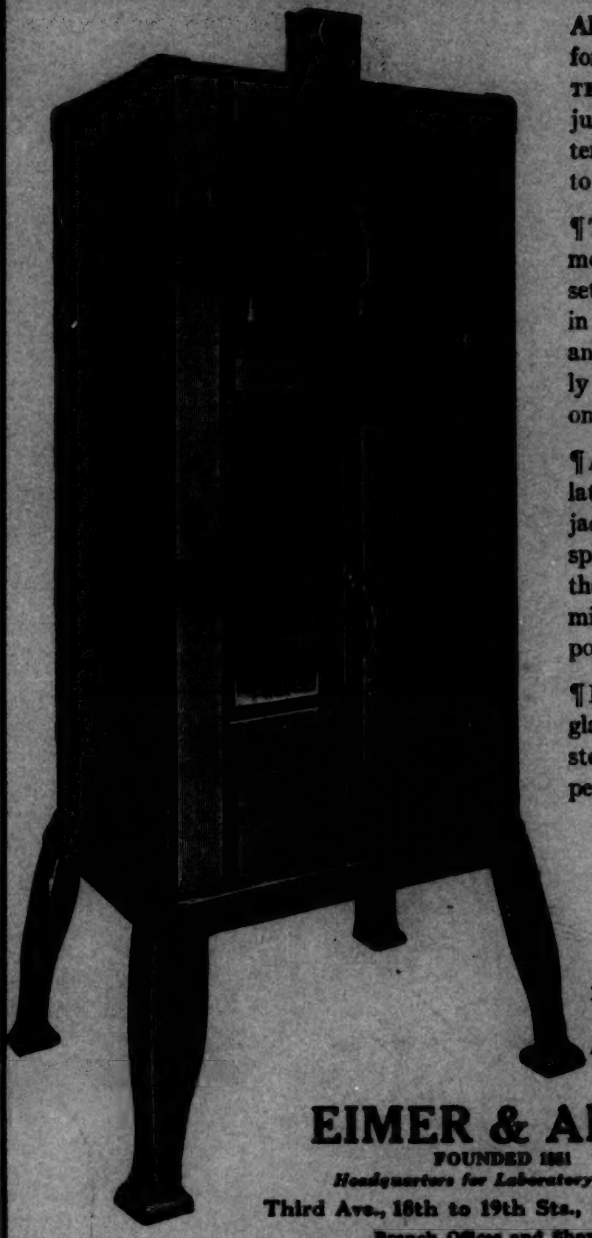

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SOME NEW CONSTITUENTS OF MILK.*

THIRD PAPER.

A NEW PROTEIN, SOLUBLE IN ALCOHOL.

BY THOMAS B. OSBORNE AND ALFRED J. WAKEMAN.

WITH THE COOPERATION OF CHARLES S. LEAVENWORTH AND OWEN L. NOLAN.

(From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.)

(Received for publication, December 6, 1917.)

From the residue obtained by concentrating the alcoholic washings from a large quantity of casein, which had been several times dissolved in dilute alkali and precipitated by dilute hydrochloric acid, we separated a not inconsiderable amount of protein which, like gliadin from wheat flour, was freely soluble in alcohol of 50 to 70 per cent by volume, but insoluble in absolute alcohol, and largely so in water containing more or less inorganic salts. The behavior of this protein towards solvents was so similar to that of gliadin and so unlike that of any protein of animal origin, hitherto described, that it seemed worth while to study it in considerable detail. That it is distinct from all of the other proteins heretofore obtained from milk was shown, not only by its behavior towards solvents and the proportion of some, at least, of the amino-acids which it yields on hydrolysis, but also by the fact that it does not react anaphylactically with any of these other proteins although it is itself highly anaphylactogenic. That this protein is an original constituent of milk and not a product of the action of alkali or other reagents used in purifying casein is shown by the fact that it can be obtained from casein which has been separated from milk simply by the addition of

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

hydrochloric acid. Owing to the fact that its acid compounds are soluble in water it can also be obtained from milk serum from which casein has been precipitated by acid and the coagulable proteins by heat.

From the purified casein which has been prepared in this laboratory we have obtained large volumes of dilute alcoholic extracts from which several hundred grams of this unique protein have been isolated in the following manner.

Casein was precipitated with dilute hydrochloric acid from centrifugated milk, filtered out, pressed, suspended in water, and dissolved, while actively stirring, by gradually adding dilute sodium hydroxide solution. From the resulting solution, which was but slightly alkaline to litmus, the casein was precipitated by dilute acetic acid. After twice reprecipitating in this way the casein was again suspended in water, dissolved by adding very dilute sodium hydroxide solution, precipitated by highly diluted hydrochloric acid, and filtered out on cheese-cloth. A sample of the filtrate when evaporated to dryness left a residue, chiefly sodium chloride, which dissolved completely in water, gave no biuret reaction, and no reduction with Fehling's solution.

The moist casein was then suspended in about an equal volume of 92 per cent alcohol, stirred for a short time, filtered out on cheese-cloth, and the extract filtered clear through paper pulp. In this way we obtained 43 liters of the first alcoholic extract, which was acid to litmus and contained 43 per cent by weight of alcohol and 350 gm. of solids, much of which was sodium chloride. The casein was again stirred up with more 92 per cent alcohol and 16 liters of a second extract were obtained containing 54 per cent of alcohol and 113 gm. of solids.

Of the first alcoholic extract 40 liters were concentrated to one-half, under diminished pressure, below 75°. The turbid concentrated extract on standing deposited on the bottom of the jars a transparent syrupy sediment, A. From this the nearly clear solution was decanted and concentrated to about 15 liters in open dishes. On cooling over night to room temperature a coherent deposit, B, formed.

A and B were dissolved in a little warm 70 per cent alcohol and the somewhat turbid solution was poured into several volumes of distilled water whereby an opaque colloidal "solution" was

produced. On adding a little sodium chloride a rapidly settling flocculent precipitate separated, leaving very little except salt in the nearly clear solution. On standing a short time the precipitate contracted to a coherent mass from which the solution was decanted almost completely.

When this precipitate was treated with about 600 cc. of absolute alcohol and warmed it yielded a solution from which a very little suspended flocculent substance was removed by filtering. The filtrate was then concentrated *in vacuo* until much protein separated in consequence of the loss of alcohol. On adding absolute alcohol and warming, a syrupy solution resulted which remained clear on cooling. This was poured into about 3 liters of absolute alcohol, whereupon most of the dissolved protein was precipitated. On adding a few drops of a strong solution of ammonium acetate the rest separated, leaving the solution clear. The precipitate was sucked out on a Buchner funnel, washed twice with absolute alcohol, and three times with absolute ether. When dried over sulfuric acid it formed a snow-white, friable mass which weighed 89 gm. and was easily reduced to a fine powder. This was designated preparation A.

Solution B, page 244, was evaporated to about 10 liters and cooled over night to room temperature. The solution was decanted from the coherent deposit which separated. The latter was rinsed with water, dissolved in warm 70 per cent alcohol, its solution filtered clear through paper pulp, and concentrated *in vacuo*, until much of the dissolved protein separated. Absolute alcohol was then added and the mixture warmed. A clear syrupy solution resulted, which remained clear on cooling. This was then poured into absolute alcohol and the protein, which separated almost completely, was sucked out, washed with absolute alcohol, and then with dry ether. When dried over sulfuric acid 46 gm. of preparation B were obtained.

The solution from which B had first separated was further concentrated to about 5 liters and, by the same procedure as employed for B, 28 gm. of preparation C were secured.

The 16 liters of the second alcoholic extract of the casein, page 244, were concentrated *in vacuo* to about 3 liters and cooled to room temperature. Nearly all of the dissolved protein separated as a coherent deposit. When subjected to the same treat-

ment as employed in making preparation B, 59 gm. of preparation D were obtained. Preparations A, B, and C represent successive fractional precipitations, while D includes all of the protein removed by extracting the casein a second time with somewhat stronger alcohol.

The following analyses show no appreciable differences between these several fractions of this peculiar protein.

Preparation	A	B	C	D
	per cent	*per cent*	*per cent*	*per cent*
Moisture at 110°	9.49	5.99	7.75	8.09
Ash	1.16	0.62	1.73	0.57
Calculated Ash- and Moisture-Free.				
	15.80	15.67	15.70	15.49
Nitrogen	15.87	15.67	15.70	15.67
		15.88		
Sulfur	0.97	0.94	0.94	1.00
Phosphorus	0.06	0.03	0.10	0.03

A composite sample of these four preparations was analyzed for carbon and hydrogen with the following results, calculated moisture- and ash-free.

Carbon	55.07	54.86
Hydrogen	7.24	7.23

A fifth preparation of this protein was made from the first and second alcoholic washings of another lot of casein which had been precipitated from about 500 liters of milk. The protein which separated after cooling the concentrated washings was dissolved in a little warm 70 per cent alcohol and its solution poured into about 50 liters of cold water. On adding sodium chloride most of the protein separated. The precipitate was collected on filters, redissolved in warm 70 per cent alcohol, and the solution filtered through paper pulp. The filtrate and washings (about 2 liters) were concentrated *in vacuo* to about 800 cc. with sufficiently frequent additions of strong alcohol to keep the protein dissolved. The concentrated solution was poured into a large volume of absolute alcohol and the resulting precipitate sucked out, washed first with absolute alcohol and then with ether. This preparation, E, when dried in warm air weighed 112 gm., and had the following composition.

	per cent	
Moisture	5.60	
Ash	1.12	

Ash- and Moisture-Free.

Carbon	54.81	
Hydrogen	7.06	
Nitrogen	15.71	15.69
Sulfur	0.89	
Phosphorus	0.28	

Comparison of the average of the above figures with the corresponding data for casein shows the alcohol-soluble protein to contain distinctly more carbon and sulfur and less phosphorus.

	Alcohol-soluble protein.	Casein.
	per cent	*per cent*
Carbon	54.91	53.11
Hydrogen	7.17	7.05
Nitrogen	15.71	15.65
Sulfur	0.95	0.82
Phosphorus	0.08	0.82
Oxygen (by difference)	21.18	22.55
	100.00	100.00

Determinations of the distribution of nitrogen, according to Hausmann's modified method, and also the proportion of basic amino-acids, according to Kossel are of interest since all known alcohol-soluble proteins of vegetable origin are characterized by a large proportion of amide nitrogen and a small proportion of basic nitrogen, especially of lysine.

The results obtained were as follows:

Partition of Nitrogen in the Alcohol-Soluble Milk Protein.

Preparation	A	B	C	D	Casein.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Amide nitrogen	1.65	1.56	1.55	1.51	1.62
	1.63	1.56	1.55	1.51	
Basic "	2.28	2.55	2.80	2.43	3.49
	2.48		2.87		
Humin "	0.26	0.21	0.24	0.22	0.21
	0.28		0.22		

Amino-Acids per 100 Gm. of Milk Protein.

	Alcohol-soluble protein. gm.	Casein. gm.
Arginine	2.92	3.81
Histidine	2.28	2.50
Lysine	3.98	5.95
Tyrosine	2.47	4.50

These results show that casein yields much more basic nitrogen, as well as more arginine, histidine, or lysine, than does the alcohol-soluble milk protein and demonstrate that these proteins are distinctly different in structure.

The alcohol-soluble protein of milk does not yield more amide nitrogen, nor less basic nitrogen, or lysine, than do most other proteins, and in these respects does not resemble the alcohol-soluble proteins of vegetable origin, although so like wheat gliadin in its solubility in relatively strong alcohol.

Further evidence that this peculiar protein has no genetic relation to casein is shown by the results of anaphylaxis experiments made by Professor H. G. Wells with a preparation which we sent him, and also with a preparation of the casein from which the alcohol-soluble protein had been extracted by dilute alcohol.

Sensitizing Dose.	gm.	*Intoxicating Dose.*	gm.	*Result.*
Casein	0.001	Casein	0.05	Died in 15 min.
"	0.001	"	0.05	" " 5 "
Alcohol-soluble protein	0.001	Alcohol-soluble protein	0.05	" " 15 "
"	0.001	"	0.05	" " 10 "
"	0.001	Casein	0.05	No symptoms.
"	0.001	"	0.05	" "

Since acid compounds of the alcohol-soluble protein are soluble in water it is probable that a further quantity is present in the milk serum from which the casein had been precipitated. Examination of the alcoholic extract of large quantities of the coagulated protein obtained by boiling the acid filtrate from the casein failed to reveal the presence of any of this protein therein.

On the other hand some was found in the filtrate from the coagulated proteins. Thus 20 liters of milk were made neutral, first to litmus and then to phenolphthalein, and the precipitates were successively filtered out. Such neutralization precipitates contain considerable protein, but numerous attempts have failed to detect the presence of the alcohol-soluble protein in them.

The filtrate from the last precipitate was made neutral to litmus with hydrochloric acid and evaporated until a large part of the lactose was removed by successive crystallizations. The mother liquor (780 cc.) was saturated with ammonium sulfate, the precipitated proteins were dissolved in water, and an equal volume of saturated ammonium sulfate solution was added which precipitated much of the protein. This fraction was dissolved in water and alcohol added to 74 per cent by volume. The precipitate produced when dried over sulfuric acid weighed 2.3 gm., Fraction I.

The alcoholic filtrate from Fraction I was treated with an excess of strong alcohol and a little ammonium acetate solution added, which caused a practically complete precipitation of the rest of the protein. This was washed by centrifuging with 92 per cent alcohol and then centrifuged with alcohol of 70 per cent (by volume) in which much dissolved. After repeating this treatment three times a turbid, colloidal solution resulted. On adding five or six drops of a strong solution of ammonium acetate some protein was precipitated. This, Fraction II, was filtered out, and when dried over sulfuric acid weighed 0.55 gm. The clear brown filtrate (200 cc.) was poured into a liter of absolute alcohol, a few drops of ammonium acetate were added, and the flocculent precipitate was centrifuged out. When dried over sulfuric acid this fraction, No. III, weighed 1.8 gm.

To determine whether Fraction III which was soluble in relatively strong alcohol contained the alcohol-soluble protein the anaphylaxis reaction was used. The results obtained by H. G. Wells were as follows:

Sensitizing Dose.	*Intoxicating Dose.*	*Reaction.*
Alcohol-soluble protein.	Alcohol-soluble protein.	Died in 15 min.
" "	" "	" " 10 "
Fraction I.	" "	None.
" I.	" "	"
Alcohol-soluble protein.	Fraction I.	"
" "	" I.	"
Fraction III.	" I.	"
" III.	" I.	"
" III.	Alcohol-soluble protein.	Severe.
" III.	" "	Died in 5 min.

These experiments show that Fraction III sensitized guinea pigs to the alcohol-soluble protein obtained from the alcoholic washings of casein, but not to Fraction I. That no anaphylaxis reaction was obtained between Fractions I and III would at first sight seem strange, for while the methods employed for separating these fractions might lead to a fairly complete separation of the alcohol-soluble protein from Fraction I a complete separation of the latter from Fraction III could hardly be expected. The failure of Fractions I and III to react with one another, however, can be explained by the fact that numerous anaphylaxis experiments with many different preparations of the proteose-like products which we have obtained from milk showed that these were mostly destitute of anaphylactogenic power. The slight reactions which were occasionally obtained can now be easily explained by the presence of the alcohol-soluble protein in some of the preparations used.

Properties of the Alcohol-Soluble Milk Protein.

The preparations of the alcohol-soluble milk protein are unquestionably acid compounds, for when 1 gm. is suspended in 50 cc. of water 2.5 cc. of 0.1 N NaOH solution are required to obtain a neutral reaction to phenolphthalein. A slightly turbid solution is thus formed which becomes distinctly more turbid when 0.5 cc. of 0.1 N HCl is added. On continuing the addition of acid up to 1.3 cc. a coarse flocculent precipitate separates leaving the solution practically clear. At this reaction very little remains dissolved.

Conversely when 1 gm. is suspended in 50 cc. of water a turbid solution results in which a not inconsiderable quantity remains undissolved. When 0.1 N NaOH solution is gradually added the protein separates in coarse flocks, nearly all being precipitated when 1.2 cc. has been added; *i.e.*, at the same reaction as that at which it separates on adding acid to the solution neutralized to phenolphthalein. When an equal volume of absolute alcohol is added most of the suspended protein dissolves after vigorous stirring. On standing, the slightly turbid solution deposits a transparent varnish-like layer which is readily soluble in warm 70 per cent alcohol, but from which a part separates on cooling

to 20°. On adding water to this transparent deposit it becomes opaque and fails to dissolve. Possibly this less soluble fraction is an acid compound which differs from the more soluble part in the kind or quantity of combined acid. The solution in alcohol of 50 per cent by volume remains clear on adding alcohol to 70 per cent at about 20°. The solubility in alcohol of various degrees of concentration depends much on the temperature. Below 30° the solubility rapidly diminishes. When suspended in water containing a very little acetic acid the alcohol-soluble milk protein dissolves completely. This solution becomes opalescent when heated to about 45° and at 80° is milk-white, but no flocculent coagulum separates on boiling. When an equal volume of absolute alcohol is then added a water-clear solution results at once. When this clear solution is poured into water an abundant separation occurs. If, instead of alcohol, sodium chloride is added, no visible separation of particles takes place, but, on standing, the solution separates into strata of differing opacity and an oily deposit slowly forms which is readily soluble in relatively strong alcohol.

Dissolved in very dilute acetic acid this protein yields a voluminous precipitate on adding potassium ferrocyanide.

It gives strong tryptophane, Millon's, and biuret reactions.

From the behavior of this protein towards water, and its reaction with potassium ferrocyanide it is evident that it is not to be considered as belonging to any group of the proteoses. Its preeminent solubility in relatively strong alcoholic solutions distinguishes it from any type of protein heretofore discovered in animal tissues or products. The possible existence of proteins of similar solubility ought to be considered whenever the complete removal of protein is necessary for the isolation of non-protein nitrogenous substances of animal origin.

STUDIES IN THE METABOLIC CHANGES INDUCED BY ADMINISTRATION OF GUANIDINE BASES.

I. INFLUENCE OF INJECTED GUANIDINE HYDROCHLORIDE UPON BLOOD SUGAR CONTENT.

By C. K. WATANABE.

(From the Laboratory of Pathological Chemistry, School of Medicine, and the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, December 13, 1917.)

It is indicated in the work of many former investigators that there exists an intimate relation between idiopathic tetany and disturbed parathyroid function. The fact that the symptoms of tetany and tetania parathyreopriva are almost identical has had experimental confirmation.

Paton and Findlay (1) confirmed the results of former workers in a series of critical investigations, in which they found that true tetany developed only after complete parathyroidectomy and that when one external parathyroid was left with its blood supply intact, no symptoms, or at most transient tremors, appear.

Many hypotheses concerning the cause of tetany have been proposed, especially from the standpoint of parathyreopriva. One states that the lack of the parathyroid produces a toxic substance in the body, or the normal function of this gland is to neutralize the toxins formed during normal metabolism. The other concerns the deficiency of some essential substance in the blood and tissues, especially calcium, which exerts an inhibition on the hyperexcitability of the nerves. It is well known that injection of calcium salts removes the symptoms for a time, but that bleeding and transfusion of saline solution (2, 3) and that bleeding followed by injection of an indifferent solution free from calcium (Ringer's solution) (4) have the same effect. These facts seem to point out that the Ca deficiency is not the true cause. Beebe's experiments in which he reduced the symptoms by injection of the extracts of parathyroids (5) and of the nucleoprotein of parathyroids (6), lead us to believe that the symptoms are due to some poison produced as a consequence of normal metabolism incident to the removal of the parathyroid. Recently Paton and Findlay (7) suggested that in tetany some toxic substances, especially guanidine

and methyl guanidine, exert a hyperexcitability on the nerves. Methyl guanidine was first isolated from normal human urine as the aurochloride by Kutscher and Lohmann (8) in 1906 and later by Engeland (9). Achelis (10) isolated it from normal dog urine as the picrolonate. The quantities found by the above three investigators correspond to about 0.007 gm. of the gold salt per liter. Koch (11) detected methyl guanidine in the urine of a dog which had been subjected to parathyroidectomy. In this case as much as 1.9 gm. per liter of the gold salt of the drug were found. In his second paper (12) he described more fully the occurrence of methyl guanidine and some of the other toxic bases in the urine in five parathyroidectomized dogs and stated that in all cases that he studied he found methyl guanidine. In those cases in which the quantity of methyl guanidine was small, other guanidine bases were present so that he considered the quantity of guanidine nitrogen to be constant in all cases. Burns and Sharpe (13), using more satisfactory methods than former workers, carefully determined the guanidine and methyl guanidine in the blood and urine of dogs after parathyroidectomy and reported a marked increase. They found an increased content of guanidine and methyl guanidine in the urine of children suffering from idiopathic tetany. They compared normal and abnormal dogs and showed an increase in guanidine of 90 per cent in blood and 40 per cent in the urine after parathyroidectomy. In the urine of children in condition of active tetany there was an increase of 500 per cent per liter over the normal. Such an increase of a spasmodic agent as guanidine suggests the cause of the development of the symptoms of tetany.

Paton and Findlay (7) demonstrated with careful observation on rabbits that the symptoms of tetania parathyreopriva are identical with those produced by the administration of the salts of guanidine and methyl guanidine.

In addition to the above workers, Underhill and others published a series of papers on carbohydrate metabolism in thyreoparathyroidectomized dogs. Hirsch (14), Eppinger, Falta, and Rudinger (15), and Pari (16) demonstrated that the assimilation limit for dextrose given by mouth or subcutaneously is lowered in thyreoparathyroidectomized dogs. Underhill and Saiki (17) also reported that thyreoparathyroidectomized dogs are incapable of utilizing subcutaneously introduced dextrose. They show a lessened oxidation or glycolysis and also a decreased ability to transform dextrose into glycogen. Underhill and Hilditch (18) observed that when the thyroids and all the parathyroids attached are removed from dogs, the ability to assimilate sugar is decreased. When the thyroid alone is extirpated this ability is not impaired. They consider that the thyroid-parathyroid mechanism stands in intimate connection with carbohydrate metabolism and that the parathyroids are the more active agents in this connection. Underhill and Blatherwick (19) found that during the tetany after thyreoparathyroidectomy, glycogen entirely disappeared from the liver and that the blood sugar content was markedly lowered. They attribute this phenomenon to the lack of parathyroid tissue since

this action may be observed only after entire parathyroidectomy and not when the removal of the thyroid leaves the parathyroids intact. Underhill and Blatherwick (20) also demonstrated that this hypoglycemia can be restored to normal temporarily and the tetany abolished for a time by the injection of calcium lactate.

From the work of Paton and Findlay (1) it appears that the symptoms of idiopathic tetany are identical with those of tetania parathyreopriva and that in both conditions there is an increased excretion of guanidine base (13) which is a well known convulsant toxin. The injection of such drugs induced the almost identical symptoms of tetania parathyreopriva (7). Therefore, it is possible that the chief cause of tetany is the production of relatively enormous quantities of guanidine bases in the body as a result of the impairment of the parathyroid function. Furthermore, if these guanidine bases are the sole source of the symptoms of tetany it seems interesting to inquire whether there is any correlation between the hypoglycemia produced by the parathyroidectomy and the guanidine content of the blood.

The present investigation deals with the influence of the injection of guanidine bases upon carbohydrate metabolism, especially the sugar content in the blood.

Methods.

Rabbits were used in this investigation. Blood was usually drawn from the ear vein before and after the subcutaneous injection of a 10 per cent solution of guanidine hydrochloride. McDanell's (21) modification of the Lewis-Benedict method was used for the estimation of sugar in the blood and Fehling's test for sugar in the urine. Duplicate determinations were done on the blood sugar to avoid technical errors. It was necessary to take some blood samples at night. In this case the specimen was immediately evaporated to dryness with the picric acid and determined colorimetrically the following morning. All specimens were evaporated to dryness with picric acid immediately on being drawn in order to avoid disappearance of sugar.

For the determination of total solids, the blood was drawn from the ear vein into a weighed crucible and dried to constant weight in an electric oven. About 2 cc. of blood were used for this determination and the sample for the blood sugar determination was taken at the same time. Comparison was made between normal rabbits and rabbits which had been injected with guanidine.

To determine the sublethal dose of guanidine hydrochloride

we used six rabbits and carefully observed the symptoms after the injection of various quantities of guanidine. The following table shows that 0.2 gm. per kilo of body weight is suitable for this purpose. As a rule all the rabbits which were used in the present work had neither food nor water for 24 hours after the injection. Immediately after the injection the rabbits usually

TABLE I.

Symptoms after Injecting Various Doses of Guanidine Hydrochloride.

Rabbit No.	Date.	Weight.	Guanidine per kilo.	Time of injection.	Symptoms and remarks.
	1917	*gm.*	*gm.*		
1	June 26	2,606	0.10	2.07 p.m.	No symptoms, recovered.
2	" 27	2,500	0.15	2.15 "	3.30 p.m. Dyspnea, twitching of head, jerking of head backwards and forwards, tremor in extremities, lying down with hind legs stretched. Symptoms disappeared by 5 p.m. Recovered.
3	" 28	2,700	0.20	2.23 "	No symptoms except slight dyspnea. Recovered.
4	" 28	2,500	0.25	10.50 a.m.	11.30 a.m. Lying down with hind legs stretched, dyspnea, chattering of teeth, jerking of head. Symptoms disappeared by 1 p.m. Died.
5	" 28	2,800	0.30	10.55 "	Quiet. No other symptoms. Died.
6	" 29	2,600	0.40	11 "	12.30 p.m. Lying down with hind legs stretched, sometimes twitching of extremities, especially hind legs. Paralysis appeared later in legs. Convulsions. Died.

run about because of the irritation produced by the injected drug. They remain quiet thereafter until the typical tetany symptoms appear—usually 1 to 1½ hours after the injection. In some cases in which small doses were administered the animal remained quiet and depressed; in other cases the animal became restless and showed slight dyspnea. When large doses were

administered the following symptoms appeared almost without exception, though usually not all in the same rabbit: dyspnea, depression, emaciation, twitching of head and legs, continuous jerking of head backwards and forwards, chattering of the teeth, fine tremor of the muscles, fibrillous tremor of extremities, opisthotonus, nystagmus following convulsion, extension of the hind legs, diarrhea, salivation, and disturbance of equilibrium.

Hypoglycemia after Injection of Guanidine Hydrochloride.

At first we administered 0.15 gm. per kilo of body weight to Rabbit 7 and 0.2 gm. per kilo of body weight to Rabbit 8 at 9.10 a.m. and kept them both under observation until the following day. The first few hours samples were taken every hour and later

TABLE II.

Hypoglycemia after the Injection of Guanidine Hydrochloride.

Rabbit No.	Date.	Weight.	Guanidine per kilo at 9.10 a.m.	Blood sugar.										
				9 a.m.	10 a.m.	11 a.m.	12 n.	1 p.m.	2 p.m.	4 p.m.	5 p.m.	8 p.m.	Next day at 9 a.m.	2 p.m.
	1917	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
7	June 30	2,220	0.15	0.101	0.100	0.122	0.124	0.100	0.098	0.085	0.077	0.073	0.099	0.095
8	July 2	2,240	0.20	0.121	0.112	0.125	0.127	0.108	0.111	0.100	0.101	0.054	0.081	

at intervals of a few hours. In Rabbit 7 mild hypoglycemia was first observed 5 hours after injection and continued until the next morning, whereas No. 8 first showed a marked hypoglycemia at 8.10 p.m. which continued until the next morning when the rabbit died in convulsions.

The following group of rabbits was used to confirm the above phenomena, the blood samples being drawn at intervals of a few hours in this case. Twelve out of twenty-one showed hypoglycemia, some of them showing a reduction of more than half the normal blood sugar. Three cases were suspected but six cases show negative results. We later confirmed the fact that in these negative cases the dose was not large enough to induce hypoglycemia. The urine of the rabbits of this group was tested but none showed any sugar. In most cases hypoglycemia appears 7 hours

after the injection and usually continues several hours. Twelve of the twenty-one died following the injection; some of them several hours after injection with typical convulsions, most of them during the following night, while a few of them died within a few days. In all cases the condition of the cage showed that the animals died in convulsions. Although the cases in which hypo-

TABLE III.

First Injection of Guanidine.

Rabbit No.	Date.	Weight.	Guanidine per kilo at 7 a.m.	Blood sugar.						
				6.55 a.m.	9 a.m.	11 a.m.	2 p.m.	5 p.m.	8 p.m.	Next day at 9 a.m.
	1917	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	Aug. 15	1,960	0.30	0.115		0.173				
21	" 8	1,520	0.25	0.093	0.098	0.080	0.058	0.056	0.053	
22	" 14	2,080	0.25	0.107	0.116	0.103	0.106	0.098	0.073	0.070
24	" 17	1,830	0.25	0.111		0.139	0.100	0.073	0.024	
31	Sept. 29	1,400	0.20	0.117			0.057	0.048		
30	" 29	1,600	0.20	0.115			0.080	0.072	0.068	
29	" 27	2,540	0.20	0.111			0.084	0.083	0.067	0.100
27	Aug. 24	2,600	0.20	0.120			0.053			
26	" 25	1,600	0.20	0.120			0.099	0.111	0.043	
18	" 5	1,520	0.20	0.113	0.143	0.132	0.103	0.057	0.039	0.086
14	July 22	1,740	0.20	0.120	0.123	0.104	0.069	0.074	0.057	0.105
15	" 23	1,520	0.20	0.100	0.105	0.094	0.067	0.081	0.073	0.095
19	Aug. 6	1,920	0.20	0.110	0.143	0.147	0.110	0.115	0.094	0.100
25	" 23	2,000	0.20	0.200			0.108	0.118	0.103	
28	Sept. 27	2,300	0.20	0.125			0.127	0.167	0.129	0.129
10	July 18	1,700	0.15	0.118	0.148	0.134	0.074	0.065	0.056	0.111
12	" 19	1,480	0.15	0.104	0.127	0.112	0.116	0.115	0.080	0.101
13	" 21	1,520	0.15	0.103	0.113	0.115	0.120	0.113	0.092	0.096
16	" 24	1,300	0.10	0.111	0.122	0.119	0.114	0.107	0.094	0.120
17	" 29	1,900	0.05	0.122	0.123	0.123	0.129	0.117	0.100	0.116
20	Aug. 7	1,440	0.05	0.103	0.105	0.102	0.107	0.099	0.102	0.104

glycemia occurs usually terminate fatally, three rabbits showing hypoglycemia recovered. The dose which was injected in this group varied from 0.05 gm. to 0.3 gm. per kilo of body weight. None of the rabbits that received under 0.1 gm. per kilo of body weight showed hypoglycemia and by careful inspection of the table it will be observed that the hypoglycemia provoked is almost

proportional to the dose of the drug administered. Rabbit 23, however, received the largest dose and this died before showing hypoglycemia. In the successive samples of blood from each rabbit we notice that in the first and second samples after injection of the drug the sugar content shows an increase over that in the sample taken before injection.

On the surviving rabbits a second injection of a little larger dose than the first was performed after time had been allowed for recovery from the first injection. In all cases except Nos.

TABLE IV.

Second Injection of Guanidine.

Rabbit No.	Date.	Weight.	Guanidine per kilo at 7 a.m.	Blood sugar.						
				6.55 a.m.	9 a.m.	11 a.m.	2 p.m.	5 p.m.	8 p.m.	Next day at 9 a.m.
	1917	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	July 8	1,880	0.25	0.111	0.142	0.154	0.140	0.120	0.096	0.123
7	" 10	2,080	0.30	0.109	0.196	0.171	0.173	0.150	0.096	0.096
9	" 14	2,260	0.25	0.152		0.125	0.091	0.095	0.098	0.143
11	" 16	1,800	0.25	0.125	0.143	0.104	0.099	0.095	0.095	0.125
12	" 31	1,280	0.25	0.111	0.102	0.093	0.104	0.111	0.087	
14	Aug. 2	1,440	0.30	0.103		0.049				
16	" 3	1,320	0.20	0.136	0.176	0.170	0.171	0.136	0.101	0.116
17	" 4	1,520	0.20	0.127	0.137	0.154	0.131	0.124	0.120	0.125
18	" 12	1,220	0.30	0.109			0.074			
19	" 13	1,400	0.25	0.107		0.154	0.125	0.122	0.107	0.111
20	" 16	1,620	0.20	0.108		0.150	0.127	0.122	0.106	0.088

14 and 18 there was no hypoglycemia—the exceptions showing hypoglycemia just before they died in convulsions.

In consideration of these facts we may say that all rabbits will also show hypoglycemia after injection of the second dose of the drug, if the dose is large enough, though in this case they will die. It is interesting to note that the second injection of a large dose of guanidine provokes, in most cases, a hyperglycemia a few hours after administration and it is more marked than that appearing after the first injection. In these animals there is no glycosuria and most of them died the following night in convulsions.

A third injection was performed on the survivors of the second injection after an interval of 1 or 2 weeks. Of these only No. 11

shows hypoglycemia and this appears just before the fatal terminal convulsion. There was marked hyperglycemia for a few hours after injection in this group of rabbits and only No. 19 recovered. There was no sugar in the urine. It is worthy of note that Rabbit 19 was injected five times at intervals of 1 or 2

TABLE V.

Third Injection of Guanidine.

Rabbit No.	Date.	Weight.	Guanidine per kilo at 7 a.m.	Blood sugar.						
				6.55 a.m.	9 a.m.	11 a.m.	2 p.m.	5 p.m.	8 p.m.	Next day at 9 a.m.
	1917	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
3	July 12	2,500	0.25	0.122		0.169	0.145	0.143	0.109	0.119
11	" 30	2,100	0.30	0.105		0.078				
16	Aug. 10	1,120	0.30	0.108	0.200	0.204	0.148	0.131	0.122	0.111
17	" 11	1,780	0.30	0.115		0.221	0.182	0.133	0.108	0.141
19	" 28	1,880	0.25	0.121	0.200	0.167	0.137	0.124	0.111	0.104

TABLE VI.

Five Experiments with Rabbit 19.

Experiment No.	Date.	Weight.	Guanidine per kilo at 7 a.m.	Blood sugar.							
				6.55 a.m.	9 a.m.	11 a.m.	2 p.m.	4 p.m.	5 p.m.	8 p.m.	Next day at 9 a.m.
	1917	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	Aug. 6	1,920	0.20	0.110	0.143	0.147	0.111		0.115	0.094	0.100
2	" 13	1,400	0.25	0.107		0.154	0.125		0.122	0.107	0.111
3	" 28	1,880	0.25	0.121	0.200	0.167	0.137		0.124	0.111	0.104
4	Sept. 11	1,850	0.25	0.113	0.133	0.084	0.064		0.091	0.088	0.127
5	" 25	1,800	0.25	0.119			0.087	0.067	0.070		0.100

weeks and that no hypoglycemia occurred the first three times. The fourth and fifth injections provoked a marked hypoglycemia.

Duration of Hypoglycemia Which Is Induced by Guanidine Administration.

Usually the hypoglycemia appears 7 hours after injection of the drug and continues several hours through the night which fact makes it impossible to take samples until the next morning when

TABLE VII.

Duration of Hypoglycemia Produced by Injection of Guanidine Hydrochloride.

Rabbit 35 (2,200 gm.).				Rabbit 37 (1,820 gm.).			
Date.	Guanidine per kilo.	Blood sugar.	Remarks.	Date.	Guanidine per kilo.	Blood sugar.	Remarks.
1917	*gm.*	*per cent*		*1917*	*gm.*	*per cent*	
Oct. 16			No symptoms until 2nd injection.	Oct. 18			
5 p.m.	0.2 at 10 p.m.	0.096		5 p.m.	0.2 at 10 p.m.	0.100	
Oct. 17				Oct. 19			
7 a.m.		0.100		7 a.m.	0.1 at 7 a.m.	0.125	Jerking backwards of head, irritable, dyspnea.
	0.1 at 9 a.m.						
10 "		0.104		9 "		0.143	
12 m.		0.111	Dyspnea, fibrillous tremor, twitching of head, sometimes convulsion.	11 "		0.145	
				2 p.m.		0.080	
				4 "		0.070	
2 p.m.		0.091		7 "		0.054	
4 "	0.05 at 4 p.m.	0.091		10 "		0.053	
7 "		0.059		Oct. 20			
10 "		0.062		9 a.m.		0.095	Sensitive but no other symptoms.
Oct. 18							
7 a.m.		0.067	Strong convulsion.	2 p.m.		0.091	
3 p.m.		0.045	At 2.30 p.m. strong convulsion, continued about 10 min. Following morning found dead.	Oct. 21			
				9 a.m.		0.064	Very sensitive, convulsion continued 10 min. At 9 a.m. took blood and killed.

the sugar content has reached the normal level. Then we tried to inject at 8 p.m. and to take our first sample early in the morning. At first three rabbits were employed and after 0.1 to 0.15 gm. per kilo of body weight was injected, no change of blood sugar was observed except in No. 19 which was doubtful. The dose was probably not large enough to produce hypoglycemia. We took four more rabbits and injected 0.2 to 0.25 gm. per kilo at 10 p.m. We took our first samples the next morning but none of them showed hypoglycemia. Another 0.1 gm. per kilo was then injected into two rabbits and from 2 p.m. until the following day, over 24 hours, marked hypoglycemia was observed. Since the above dose is enough to induce hypoglycemia, the inference is drawn that the manipulation in taking the blood or the excitation of the animals accelerates the production of hypoglycemia.

The Relation of Total Solids in the Blood to the Blood Sugar Content.

The question arose whether this diminution in blood sugar was due merely to the dilution of the blood or to the effect on the sugar-regulating mechanism. To make this point clear the estimation of total solids of the blood was made.

The following table shows that when samples were taken from normal rabbits during 12 hours the variation in sugar content was much smaller than the variation in total solids.

After a few days the same rabbits were used for injecting the

TABLE VIII.

Normal Variation of Total Solid and Sugar Percentage in Blood.

Rabbit No.	Date.	9 a.m.		2 p.m.		7 p.m.	
		Sugar.	Solid.	Sugar.	Solid.	Sugar.	Solid.
	1917	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
25	Aug. 19	0.116	16.4	0.111	16.4	0.114	15.8
26	" 20	0.115	17.4	0.105	16.0	0.108	15.1
26	" 22	0.125	15.3	0.126	14.8	0.122	13.8
27	" 21	0.114	18.8	0.108	18.3	0.110	16.6
28	Sept. 21	0.123	16.1	0.123	15.4	0.125	15.3
29	" 21	0.104	16.6	0.111	15.8	0.103	14.9
30	" 23	0.106	16.2	0.111	15.5	0.116	14.8
31	" 23	0.111	15.2	0.114	14.8	0.105	14.5
Average.		0.114	16.5	0.114	15.9	0.113	15.1

guanidine. One other rabbit was used also which later was injected twice more. One sample for comparison was taken before injection. In some animals the dose is not large enough to produce hypoglycemia. In those animals in which hypoglycemia appeared the total solids in the blood remained normal. It is worthy of note that the variation in total solids in the same rabbit is practically the same in experiments tabulated in Table VIII as in those in Table IX. In Rabbit 19 hypoglycemia appeared only after the second and third injection when the total solids remained the same as after the first injection.

TABLE IX.

Variation of Total Solid and Sugar Percentage in Blood before and after Guanidine Injection.

Rabbit No.	Date.	Guanidine injection per kilo of body weight at 7 a.m.	7 a.m.		2 p.m.		3 p.m.		4 p.m.		5 p.m.		8 p.m.	
			Sugar.	Solid.	Sugar.	Solid.	Sugar.	Solid.	Sugar.	Solid.	Sugar.	Solid.	Sugar.	Solid.
	1917	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
25	Aug. 23	0.20	0.200	15.1	0.108	16.3					0.118	15.5	0.103	14.4
27	" 24	0.20	0.120	16.9	0.053	18.5								
28	Sept. 27	0.20	0.125	15.2							0.167	14.8	0.129	14.5
29	" 27	0.20	0.111	16.5	0.084		0.087	16.0			0.083	15.4	0.067	15.0
30	" 29	0.20	0.115	16.0	0.080	15.8					0.072	14.6	0.068	13.9
31	" 29	0.20	0.117	15.5	0.057	15.9			0.048	17.5				
19	Aug. 24	0.25	0.121	15.4	0.137	15.3					0.124	14.4	0.111	13.7
19	Sept. 11	0.25	0.113	16.4	0.064	16.2					0.091	15.2	0.088	15.4
19	" 25	0.25	0.119	16.5					0.069	17.3	0.070	16.9		

Tetania parathyreopriva and idiopathic tetany are almost identical in the character of their symptoms, such as electric excitability, increased mechanical excitability, depression, emaciation, spasticity, tremor, convulsion, laryngeal spasm, etc., according to many workers. According to Burns (22) there is a striking resemblance between the metabolic evidences of tetania parathyreopriva and the injection of guanidine. Burns and Sharpe (13) demonstrated the increased guanidine base in the urine of children which manifested symptoms of idiopathic tetany and also in the urine and blood of animals which had symptoms of tetania parathyreopriva. Moreover, the serum of para-

thyroidectomized animals acts upon the muscle of frogs in the same way as do dilute solutions of guanidine (23). The administration of the salts of guanidine to animals induces symptoms almost identical with those of tetania parathyreopriva (7). The above evidence strengthens the view that a function of the parathyroids is the regulation of the metabolism of guanidine in the body and that the guanidine thus produced controls the tone of the muscle as mentioned by Paton and Findlay (24).

The results of the present investigation show that the hypoglycemia which is manifested in tetania parathyreopriva, as shown by Underhill and Blatherwick (19), can also be produced by the injection of guanidine hydrochloride. The hypoglycemia manifested after parathyroidectomy may be a secondary manifestation of guanidine poisoning.

The hyperglycemia induced several hours after administration of the drug, especially after the second and third injection, may be considered to be due to the disturbance of respiration since it is well known that dyspnea induces an increase in the sugar content of the blood.

CONCLUSIONS.

The administration of guanidine hydrochloride into rabbits induces symptoms which are almost identical with those of tetania parathyreopriva. At the same time hypoglycemia is induced which also occurs after the removal of the parathyroid. Therefore the hypoglycemia resulting from the lack of parathyroid may be due to the action of guanidine which is increased in the blood under these conditions.

The hypoglycemia induced by injection of guanidine is not due to the diminution of total solids or to the dilution of the blood.

The author acknowledges his indebtedness to Professor F. P. Underhill for suggestions and advice in carrying out this work.

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MATHEMATICAL FORMULAS AND ACID EXCRETION.

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The application of mathematical formulas in the attempt to elucidate biological processes has lately furnished us with laws, quantitative relationships, constants, and indices which might seem to leave little to be desired. Some of us, however, have been inclined to question the value of certain of these laws, particularly as applied to the excretion of a variety of substances by the kidney, not only on account of the positive contrary evidence brought forward, but also on the ground that we believe it improbable that our present knowledge is sufficient to permit us to express the complexities of kidney activity with mathematical accuracy.

An analysis of the data presented in the recent paper of Fitz and Van Slyke[1] on acid excretion affords a specific basis for criticism of one such mathematical relationship. In this paper the authors search for a "quantitative relationship . . . . between the alkali reserve of the blood plasma, as measured by the combining power for CO_2, and the rate of acid excretion by the kidneys," and arrive at the conclusion that the combined formula developed by Ambard for urea and for chloride excretion is applicable here in the form

$$\text{Plasma } CO_2 = 80 - \sqrt{\frac{D}{W}}\sqrt{C}$$

where D is the excretion rate for acid plus ammonia, C their concentration in the urine, and W the weight of the individual. They adopt this form as an expression of the true quantitative relationship only after trials of various modifications, concluding

[1] Fitz, R., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 389.

that it holds "more consistently than any other expression which could be found in the literature or devised." In their development they note, for instance, that the fourth root of C has been retained because the square root gave too much and the sixth root too little influence to the factor C. Now since it does not appear from any ordinary inspection of the values of C given that it can have any appreciable influence, it would seem logical to revise the formula, replacing C by a constant. Thus it is possible to arrive at the formula

$$\text{Plasma } CO_2 = 80 - 5\sqrt{\frac{D}{W}}$$

whose calculated values for plasma CO_2 for the 76 observations given show an average deviation from the found values of 5.45 volumes per cent compared with 6.99 volumes per cent by the original formula. That is to say, the results average 1.5 volumes per cent better if instead of using the fourth root of C we use the figure 5. Therefore we must conclude that the "influence" of C in this formula is negligible, and that consequently the corollary statement made by the authors that "other factors being the same, the amount of acid excreted in excess of mineral bases is increased, on the average, as the square root of the volume of urine" must also be revised; for it is apparent that as far as we may conclude from this equation the acid excretion is quite independent of the volume of urine.

We may further omit W, and devise the form

$$\text{Plasma } CO_2 = 80 - 0.7\sqrt{D}$$

which will also give results more closely approximating the found values of CO_2 than those derived from the Ambard type of formula.

Or if we wish to make use of the ammonia excretion rate only, it will be found that from the equation

$$\text{Plasma } CO_2 = 80 - 0.9\sqrt{NH_3}$$

(where NH_3 is the amount of 0.1 N ammonia excreted per 24 hours) we can likewise calculate the index of blood bicarbonate with considerably greater precision and with much less mathe-

matical effort. And so on. It would no doubt be possible to evolve many other forms equally applicable. The following table summarizes the comparative merits of the Ambard type of formula and the three modifications suggested.

Formula.	Average deviation. *vol. per cent*	No. of errors greater than 10 volumes per cent.
$CO_2 = 80 - \sqrt{\frac{D}{W}}\sqrt{C}$	6.99	15
$CO_2 = 80 - 5\sqrt{\frac{D}{W}}$	5.45	11
$CO_2 = 80 - 0.7\sqrt{D}$	5.85	12
$CO_2 = 80 - 0.9\sqrt{NH_3}$	6.35	11

While it is thus apparent that many formulas can be derived which show a certain degree of applicability to the facts of acid excretion, it does not at all follow that they are to be regarded as true quantitative relationships. Certainly before we accept any formula as such we must justify it further than by the mere statement that "the margin of error to be accepted in this instance appears to be about 10 volumes per cent of plasma CO_2." For certainly if the relationship be a true one this margin of error must bear some fairly close relation to the deviation produced by possible errors in the original observations. Suppose, then, in the original formula above, we allow an error of 5 per cent in determining urinary acidity, a similar error in determining ammonia, 2.5 per cent error each in measuring urinary volume and the weight of the individual, and 6 per cent error in determining plasma bicarbonate, all of which are much greater than the errors of the methods involved. If such maximal errors occurred in *every* urinary, weight, volume, and CO_2 determination in the entire series and always occurred in such a manner as to make their effects additive, the error deviation of the calculated from

the found CO_2 would be less than 5 volumes per cent,[2] or considerably less than the *average* deviation found by the best of the above laws.

From this brief analysis we must therefore conclude that the Ambard type of formula cannot be regarded as a quantitative relationship between the variables of acid excretion, and that no mathematical expression so far devised can lay claim to even a moderate degree of accuracy as such. Furthermore, it seems obvious that such formulas constantly give the impression of mathematical accuracy where none exists, and that their use is apt to prove misleading to the many who are inclined to accept them without question.

SUMMARY.

In criticism of the application of the Ambard type of formula to acid excretion, it is shown that:

1. Substitution of constants for certain of the variables leads to an improvement in the calculated results.

2. The average deviation of calculated from found values of plasma CO_2 is much greater than would result from maximal additive errors in all the determinations involved.

[2] Under the influence of the assumed errors the quantity $\sqrt{\frac{D}{W}\sqrt{C}}$ becomes $\sqrt{\frac{1.05\,D}{0.975\,W}\sqrt{\frac{1.05\,D}{0.975\,W}}}$, simplifying which we obtain

$$\left(\frac{1.05}{0.975}\right)^{\frac{1}{4}}\sqrt{\frac{D}{W}\sqrt{C}}, \text{ or } 1.06\sqrt{\frac{D}{W}\sqrt{C}}.$$

From this source the error in volumes per cent of calculated CO_2 will thus be 6 per cent of $\sqrt{\frac{D}{W}\sqrt{C}}$. Allowing also a 6 per cent error in the CO_2 determination, the greatest possible maximal deviation will be $0.06\left(CO_2 + \sqrt{\frac{D}{W}\sqrt{C}}\right)$, and since by the given law $CO_2 + \sqrt{\frac{D}{W}\sqrt{C}} = 80$, this becomes 4.8 volumes per cent.

STUDIES OF ACIDOSIS. X.

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The present paper includes a reply to Barnett's (1) critique in the preceding article and a summary showing the nature of the results obtained with the methods for estimating alkaline reserve from urine and alveolar air analyses.

Fitz and the writer (2) succeeded recently in demonstrating a quantitative relationship between the alkaline reserve and the excretion of acids in excess of fixed base, as measured by ammonia plus titratable acid in the urine. The empirical formula utilized to demonstrate the relationship was, plasma $CO_2 = 80 - \sqrt{\frac{D}{W}}\sqrt{C}$, D representing the cc. of 0.1 N titratable acid and ammonia excreted per 24 hour time unit, and C (concentration) the amount excreted per liter.

Barnett states the belief that this formula does not express the relationship between plasma bicarbonate and acid excretion with sufficient accuracy to justify its use. The reason for this opinion is that the results show that the average error in calculating the plasma bicarbonate from the excretion exceeds that which would result from maximal analytical errors in all of the determinations involved. This seems to us insufficient ground for the criticism. The essential question, for our purpose, is not whether the plasma bicarbonate can be estimated from urine excretion with no error except that of the chemical determinations, but whether the total sum of the errors of analysis, individual variation in kidney function, and fault in the empirical formula is within such limits that the excretion may be of any assistance in estimating the alkaline reserve when conditions prevent the direct determination of the latter in the blood.

We have given in Papers IV (2) and VI (3) data which show the limits of accuracy encountered in estimating plasma bicar-

bonate from acid excretion in practically every form of diabetic acidosis. It does not appear to us that a valid reason has been advanced for modifying the conclusion based on those data; *viz.*, that the ammonia plus acid excretion is quantitatively related to the bicarbonate deficit in the blood, and that the relationship is sufficiently uniform to be useful in estimating this deficit when the limitations of accuracy, as shown by our data, are taken into consideration.

Barnett's claim that all the variables of the formula except the two most important ones, the blood bicarbonate and ammonia excretion, may be replaced by a constant without significantly diminishing the average accuracy of the formula, would likewise appear to us, even if entirely justified, to detract nothing from the correctness of the above conclusion.

Any simplification in the calculation not resulting in loss of accuracy would be an improvement, however, and therefore it is desirable to examine somewhat closely the formula minus one, two, and three of its variables in order to decide how many of them may be deleted without lessening the reliability of results.

Concerning the dropping of C, thereby simplifying the formula

$$\text{Plasma } CO_2 = 80 - \sqrt{\frac{D}{W}}\sqrt{C} \text{ to, Plasma } CO_2 = 80 - 5\sqrt{\frac{D}{W}},$$

we agree with Barnett that it makes the equation not only simpler, but also measurably more accurate and is therefore in every way desirable.[1] In preliminary experiments in which we tested several formulas before one was chosen to apply systematically, the value of C appeared to have an appreciable effect, although much less than that of D; *i.e.*, other conditions being the same, a greater volume of urine appeared to carry out somewhat

[1] From the negligible average influence of C it appears that, at least as long as either normal or exceptionally high volumes of urine are excreted (which was the case with all our patients), variations in the volume are of no effect on the acid excretion. We are not, however, prepared to follow this conclusion as to the non-effect of C to its logical limit and state that in a patient with severe diabetic acidosis flushing with water is of no value. A liter of urine is not an abnormally small amount in a normal adult, but it could carry out only about 15 gm. of β-hydroxybutyric acid, if, as indicated by the data of Magnus-Levy and others, this acid is never excreted in concentration greater than 1.5 per cent. To remove the

more acid than a smaller volume. We accordingly indicated this effect by introducing C into the formula, and indicated its comparatively minor importance by using its fourth root. The formula thus involved enabled us to solve our main problem; *viz.*, the question as to whether any quantitative relationship could be demonstrated between fall in alkaline reserve and rise in ammonia and acid excretion. The formulas which had been discarded in the preliminary tests we did not afterwards apply to the main body of our data. It is fortunate that Barnett has now retested the formula without C on our published data, and shown that this variable may be neglected. With the formula thus simplified it becomes possible, as indicated in the accompanying table, to interpret the 24 hour excretion of acid plus ammonia directly into terms of acidosis with the use of no more elaborate terms than $\frac{D}{W}$, or cc. of 0.1 N acid plus ammonia per kilo.

Barnett also calculates from the results of Paper IV that but little is gained in average accuracy by including variations in the body weight in the estimation. The data of Paper IV, however, taken without those of Paper VI, are not suited to decide statistically the question of the influence of body size. Of the 65 determinations on diabetics reported in Paper IV, 29 are on a single patient of 50 kilos weight, and of the others, only one determination was made on a subject of less than 37 or more than 50 kilos weight who had a marked acidosis without bicarbonate dosage. In this one, a boy of 12 in actual coma, both the original $\sqrt{\frac{D}{W}}\sqrt{C}$ and the 5 $\sqrt{\frac{D}{W}}$ formula indicate a plasma CO_2 of 30 per cent, which is a severe acidosis, though not so severe as that shown both by clinical con-

much larger amounts sometimes formed, excretion of several liters of urine per 24 hours appears necessary. The formation of large amounts of acetone bodies, or of the sugar which accompanies them, apparently acts as a diuretic, and secures the necessary excretion. For example, Magnus-Levy reports that when a patient on 2 successive days excreted 109.5 and 157 gm. of organic acid calculated as hydroxybutyric, the corresponding urine volumes were 8.0 and 9.2 liters (Magnus-Levy (4), p. 182). Similar high excretions are seen in the data of Papers IV and VI when acid excretion, as indicated by the ammonia plus titratable acid, was high.

dition and plasma CO_2 of 14 per cent. If the body weight were neglected in the calculation, however, and Barnett's $0.7\sqrt{D}$ formula used, the excretion would indicate a plasma CO_2 of 46, or almost no acidosis. Similar magnification of error is introduced in attempting to interpret the excretion data regardless of body weight in the two patients of less than adult size with acidosis reported in Paper VI. These are No. 3, a boy of 12 with intense acidosis, and No. 5, a boy of 13 with severe acidosis. That an allowance for body size must be made in interpreting the rate of formation or excretion of any metabolic product is a generalization so well founded that previous discussion of it seemed unnecessary.

Elimination of a third variable, the titratable acid, from the calculation, would apparently be a further step backwards. It is true, as exemplified by our own data, that ammonia and titratable acid in diabetic urine as a rule rise and fall together, the ammonia being usually two to three times the titratable acid. The ratio $\frac{0.1\ \text{N}\ NH_3}{0.1\ \text{N acid}}$ is by no means constant, however, varying from 0.3 to 5.0 in diabetic and normal urines, so that the titratable acid sometimes exceeds the ammonia. Since both ammonia and titratable acid indicate excretion of acid in excess of fixed base, it is does not seem logical to determine the one and neglect the other. The result of neglecting the titratable acid is apparent, except in the case of the one diabetic who was chosen for continuous observation, in a decided increase in the average error.

Average Errors. Data of Paper IV.

Formula.	Table I. 11 normal persons.	Table II. 36 different diabetics.	Table III. 29 observations on 1 diabetic.
$CO_2 = 80 - 5\sqrt{\frac{D}{W}}$	2.5	5.4	5.7
$CO_2 = 80 - 0.9\sqrt{NH_3}$	4.6	7.4	5.7

A practical additional reason for determining titratable acid as well as ammonia is that it protects against a false diagnosis of acidosis which might be made from the ammonia alone in urines

that have undergone bacterial decomposition, either in the bladder as the result of cystitis, or outside the bladder as the result of preservation with insufficient antisepsis. As long as the ammonia formation leaves the urine still acid, it does not much alter the NH_3 + acid figure, since CO_2, of the ammonium carbonate formed by bacterial action on urea, escapes, while the ammonia remains and neutralizes approximately an equivalent of acid. The net effect of an increase of ammonia is therefore an approximately equal decrease of titratable acid, with no significant influence on the resultant sum of the two.

Our data indicate furthermore that the ratio $\frac{0.1\ N\ NH_3}{0.1\ N\ acid}$ may be used as a fairly sensitive indicator of ammoniacal decomposition. In the urines of Paper IV, which were all analyzed while perfectly fresh, the ratio varies from 0.3 to 4.8 averaging 1.6 in normal men and 2.3 in diabetics. In only one case was a value of 4.1 exceeded. It appears therefore that when the ammonia exceeds four or five times the titratable acid there is ground for suspecting the origin of a measurable portion of the ammonia in bacterial action. Although the 24 hour urines reported in Paper VI are believed to have been collected with at least ordinary care and were preserved with toluene, it will be seen from the results that in these urines the ammonia:acid ratio averages higher (about 4.1) than in the quickly collected and analyzed specimens of Paper IV, and frequently exceeds the maximum of the short-time urines, at times rising as high as 8 or 9. In no case had decomposition gone so far as to neutralize all or nearly all of the titratable acid, so that the results, based on the sum of ammonia and titratable acid, could not have been significantly affected. The frequency of high ammonia:acid ratios in the 24 hour urines nevertheless indicates the readiness with which decomposition may occur in 24 hour specimens even when collected with routine precaution.

For the above reasons we believe that when diabetic acidosis must be estimated from acid excretion, the most satisfactory formula at present available for expressing the results in terms of alkaline reserve is

$$\text{Plasma } CO_2 = 80 - 5\sqrt{\frac{0.1\ N\ (\text{acid} + NH_3)\ \text{per 24 hours}}{\text{kilos body weight}}}$$

and that neither the titratable acid nor body weight may be neglected without increasing the chance of error in the estimation.

As, thanks to Barnett, the acid excretion formula is simplified to the above by elimination of one unnecessary variable, and as Palmer and Van Slyke in Paper IX (5) have published data which add the bicarbonate retention to the indirect acidosis tests that have been composed with the direct, it appears desirable to revise accordingly the summarizing table on page 412 of Paper VI. In the following table we have therefore substituted the simpler expression for acid excretion and have added the data for the bicarbonate retention test. We have also indicated the chief fallacies to which, according to the results published in the present series of papers, each indirect test is liable when applied to the detection of diabetic acidosis.

It should be noted that the data obtained from kidney and lung excretion as measures of alkaline reserve in diabetic acidosis do not necessarily hold for other types of acidosis. In nephritis, for example, the two tests based on kidney excretion become fallacious, while Peters (6) has recently shown that in cardiac dyspnea and in conditions involving great diminution of lung capacity the mechanics of respiration are so disturbed that the alveolar carbon dioxide ceases to be an approximate measure of blood bicarbonate. The indirect tests may be trusted as approximate indicators of alkaline reserve only in conditions where they have been previously tested by comparison with the blood bicarbonate. We have made this comparison in diabetes, but the results do not hold for other pathological conditions.

SUMMARY.

Acid excretion as a measure of diabetic acidosis is, according to present data, most significantly expressed in terms of ammonia plus titratable acid per unit of body weight. The average error involved in estimating alkaline reserve from acid excretion is, as shown by Barnett, appreciably reduced by simplifying our original empirical formula, plasma $CO_2 = 80 - \sqrt{\frac{D}{W}\sqrt{C}}$, to plasma $CO_2 = 80 - 5\sqrt{\frac{D}{W}}$. The absolute difference in the re-

Condition of subject.	Actual bicarbonate reserve. Plasma bicarbonate CO_2, reduced to 0°, 760 mm.	Corresponding results of indirect tests for acidosis.					
		24 hour excretion* of 0.1N acid + NH_3.		Carbon dioxide of alveolar air.		Sodium bicarbonate required to turn urine alkaline.	
		(*a*) Co. per kg. (*b*) Approximate cc. per 60 kg. person.	Reliability in diabetes.	(*a*) Mm. tension. (*b*) Approximate per cent.	Reliability in diabetes.	(*a*) Gm. per kg.‡ (*b*) Approximate gm. for a 60 kg. person.	Reliability in diabetes.
	vol. per cent	*cc.*					
Normal resting adult. Extreme limits of bicarbonate reserve.	80–53	(*a*) 0–27 (*b*) 0–1,600	Good.	(*a*) 53–35 mm. (*b*) 6.8–4.7 per cent.	May indicate some acidosis in its absence.	(*a*) 0–0.5 (*b*) 0–30	May indicate acidosis in its absence.
Mild acidosis, no pronounced symptoms.	53–40	(*a*) 27–65 (*b*) 1,600–4,000	Good.†	(*a*) 35–27 mm. (*b*) 4.7–3.6 per cent.	May indicate more acidosis than is present.	(*a*) 0.5–0.8 (*b*) 30–50	May indicate much more acidosis than is present.
Moderate to severe acidosis. Symptoms may be apparent.	40–30	(*a*) 65–100 (*b*) 4,000–6,000	Liable† to considerable error in either direction.	(*a*) 27–20 mm. (*b*) 3.6–2.7 per cent.	Good.	(*a*) 0.8–1.1 (*b*) 50–65	" "
Severe acidosis. Symptoms of acid intoxication.	Below 30	(*a*) Over 100 (*b*) " 6,000	" "	(*a*) Below 20 mm. (*b*) 2.7 per cent.	"	(*a*) Over 1.1 (*b*) Over 65	" "

* Measured either in 24 hour urine or on specimen from shorter period calculated to 24 hour basis.

† After bicarbonate administration likely to indicate more acidosis than is present.

‡ The figures tabulated in this column also indicate the doses of bicarbonate necessary to restore the alkaline reserve to normal from acidosis of the severity indicated by the corresponding plasma CO_2 figures in the first column.

sults calculated by the two equations is, however, so small that the remarks on the range of error in such calculations made in Papers IV and VI hold with essentially equal force when the simplified formula is used. Further simplification, by neglecting the body weight or titratable acid as suggested by Barnett, decreases the accuracy of the estimation.

For practical purposes the acid excretion may, without going through the calculation of the formula, be interpreted directly into terms of clinical severity of acidosis, as indicated in the table; *e.g.*, an excretion exceeding 27 cc. of 0.1 N ammonia plus acid per kilo indicates acidosis, which usually becomes critical if the excretion approaches 100 cc. per kilo.

The relationships of the plasma bicarbonate to acid excretion, alkali retention, and alveolar carbon dioxide tension are summarized for reference in a table, wherein are also indicated the chief errors to which, according to the data of Papers IV, VI, and IX of this series, the three latter determinations are subject as measures of diabetic acidosis.

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4. Magnus-Levy, A., *Arch. exp. Path. u. Pharm.*, 1899, xlii, 149.
5. Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.
6. Peters, J. P., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 118.

THE EFFECTS OF ELECTROLYTES ON GELATIN AND THEIR BIOLOGICAL SIGNIFICANCE.

I. THE EFFECTS OF ACIDS AND SALTS ON THE PRECIPITATION OF GELATIN BY ALCOHOL.

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The biologist can hardly hope to find a complete explanation of the complicated effects of electrolytes upon protoplasm until the chemist can give him an explanation of their almost equally complicated effects upon the purified constituents of protoplasm. In order to obtain more complete information concerning the effects of electrolytes upon proteins, the precipitation of gelatin by alcohol has been studied. This property of gelatin is more simple to measure and more sensitive to small variations in the salt content of the solution than its viscosity, swelling, or temperature of gelation. This method has yielded, therefore, more complete data than have hitherto been obtained. Pending a more detailed inquiry into the reactions involved, it is deemed advisable to publish the facts obtained, omitting the theoretical interpretation.

Description of the Method.

The method which was worked out for the investigation has already been briefly described[1] together with some of the results which are of particular interest to the biologists. It consists essentially in adding 95 per cent alcohol to 5 cc. of the gelatin-electrolyte mixture until an opaque precipitate is produced. The sharpness of the end-point varies characteristically with the particular electrolyte and the particular concentration used but can ordinarily be determined to within 0.1 cc., which is

[1] Fenn, W. O., *Proc. Nat. Acad. Sc.*, 1916, ii, 534, 539.

accurate enough for the purpose. Two determinations were made for each point and the average was taken as the true value. The test-tubes containing the samples to be titrated with alcohol were kept at constant temperature for some hours before using in order to reach equilibrium. The time allowed for this and the temperature chosen varied considerably in the different experiments but, in plotting, only curves which are comparable in all essential respects are placed together, unless mention is made to the contrary. In mixing, the gelatin was dissolved in water at 90°C. and 5 cc. of the resulting solution were mixed with 5 cc. of the solution of the electrolyte to be investigated. The two halves, of 5 cc. each, of these mixtures were used for the two determinations of each point. Three grades of commercial gelatin were used throughout. Gelatin A, which was used in all except the earlier experiments, set at a lower concentration and gave a clearer solution than gelatin B or C. The concentration of the gelatin was so chosen (2 or 3 per cent) that it would not set at the temperature of the experiment. Throughout this paper, the number of cc. of 95 per cent alcohol necessary to produce an opaque precipitate in 5 cc. of the gelatin-electrolyte mixture will be spoken of as the "alcohol number" for the sake of brevity. In interpreting the results, it should be remembered that in titrating, both the gelatin and the electrolyte are being diluted, and the solution is being cooled by the addition of the alcohol. The alcohol number is ordinarily increased by a rise in temperature and by dilution of the gelatin. The cooling effect of the alcohol could be avoided by allowing the gelatin to reach equilibrium at room temperature, but sharper end-points are obtained by using stronger gelatin. This necessitates a higher temperature to prevent setting. If, in titrating, the alcohol is run in too rapidly, the value obtained for the alcohol number will be too high as the precipitate takes a certain short time to form. This error is important only in the case of NaOH and HCl and similar electrolytes. The gelatin is stirred after each addition of alcohol. The end-point is defined as the point where a pencil held behind the test-tube can no longer be seen.

The alcohol number is ordinarily increased by a rise in temperature but in the presence of Na_2SO_4 in concentrations greater than 0.2 M and NaCl M or stronger, the reverse is true.

The results obtained by this method may be presented under the following four headings: (I) The effect of single electrolytes. (*a*) Acids and alkalies; (*b*) salts. (II) The effect of combinations of salts with acids and alkalies. (III) The effect of combinations of salts. (IV) The precipitation of gelatin by mixtures of electrolytes without alcohol. Each of these will be presented in a separate paper.

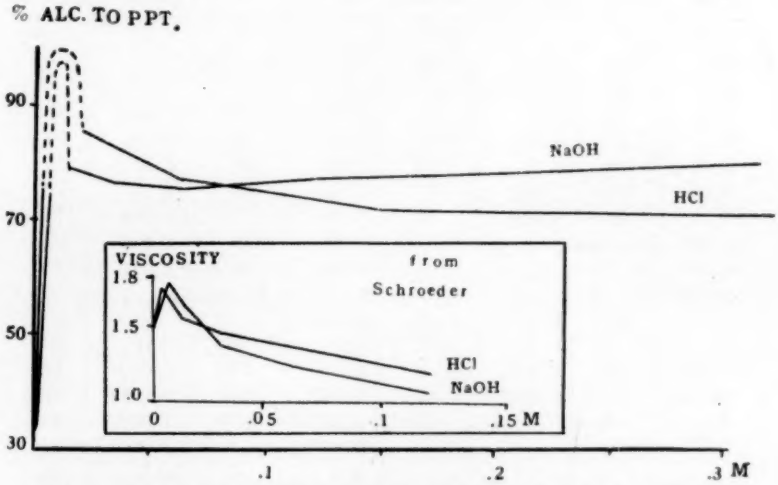

FIG. 1. Curves showing the effect of NaOH and HCl on the precipitation of gelatin by alcohol. The ordinates represent the concentrations of alcohol in the solution at the point of precipitation, and the abscissæ the concentrations of HCl and NaOH at that point. The dotted lines indicate that no precipitate could be obtained no matter how much alcohol was added. The insert shows von Schroeder's curves for the viscosity of gelatin sols, showing that the alcohol maximum corresponds to a viscosity maximum.

a. Acids and Alkalies.

The presence of acids and alkalies in gelatin hinders the precipitation by alcohol. With strong acids and alkalies, like NaOH and HCl, this effect passes through a maximum.

The effects of NaOH and HCl are shown in Fig. 1, where the concentration of alcohol at the point of precipitation is plotted (ordinates) against the concentration of NaOH or HCl at the end-

point (abscissæ). The maximum in the curves corresponds to a point of maximum swelling and maximum viscosity. Von

TABLE I.

Effect of HCl and NaOH on the Precipitation of Gelatin by Alcohol.

HCl				NaOH			
Concentration of HCl at start.	Alcohol to precipitate.	Concentration of HCl at end-point.	Alcohol (by volume) at end-point.	Concentration of NaOH at start.	Alcohol to precipitate.	Concentration of NaOH at end-point.	Alcohol (by volume) at end-point.
M	*cc.*	M	*per cent*	M	*cc.*	M	*per cent*
12.2	55	1.0	85	4.9	No ppt.		
6.1	30	0.85	79	3.26	" "		
3.05	19	0.61	72	2.17	33	0.38	81
1.52	18	0.32	71	1.45	29	0.26	79
0.76	19	0.15	72	0.96	28	0.17	78
0.38	25	0.063	77	0.64	26	0.12	77
0.19	50	0.018	85	0.32	23	0.064	75
0.095	No ppt.			0.16	24	0.032	76
0.048	" "			0.08	30	0.014	79
0.024	" "			0.04	No ppt.		
0.012	23	0.0021	75	0.02	" "		
0.006	9	0.002	57	0.01	16	0.0027	69
0.003	6	0.0013	47	0.005	8	0.0021	54
0.0015	4	0.00075	37	0.0025	6	0.0012	47.5
0.0	3		32	0.00125	5	0.0007	43
				0.000625	4.5	0.00035	41
				0.0	3.2	0.0	33

5 cc. of the HCl and NaOH solutions, in the concentrations noted in the first columns, were placed in each tube. 1 cc. of 7 per cent gelatin B was then added to each. HCl and NaOH are so effective in preventing precipitation that the titration with alcohol must be carried on slowly in order to allow the precipitate time to form. In order to save time, all the tubes were titrated simultaneously. The same number of cc. of alcohol was added to each in turn until precipitation occurred. The experiments were performed at room temperature. The decrease of precipitability in high concentrations is due to decomposition of the gelatin. For purposes of plotting, the concentrations of HCl, NaOH, and alcohol at the point of precipitation are calculated. These data are plotted in Fig. 1.

Schroeder's curves for the viscosity of gelatin sols are plotted on the insert for comparison.[2] Their essential similarity to the alcohol curves is undoubted.

[2] von Schroeder, P., *Z. physikal. Chem.*, 1903, xlv, 75.

Similar curves have been worked out for various other acids. Lactic, trichloroacetic, acetic, and formic acids give curves which rise more gradually than the HCl curve. It is impossible to follow them through the maximum as no precipitate can be obtained. Phosphoric acid gives a sharp fall after the maximum which can be followed, but no precipitate can be obtained at the maximum. Tartaric acid and sulfuric acid rise slowly to a comparatively low maximum and do not drop thereafter. In general, the weak acids have less effect and show either no maximum or less maximum than the HCl curve. The small effect of H_2SO_4, compared to HCl and HNO_3, is probably due to the effect of the bivalent SO_4 anion.

The fact that acids and alkalies prevent the precipitation of proteins by alcohol was shown by Pauli and Handovsky[3] and by Schorr,[4] although in neither case was the complete course of the curve worked out. Pauli and collaborators,[5] Lacqueur and Sackur,[6] and Zoja[7] have shown that acids and alkalies increase the viscosity of protein sols, and von Schroeder[2] has shown the same for gelatin sols. The swelling of gelatin has been measured by Ostwald,[8] Procter,[9] Fischer,[10] and Ehrenberg.[11] The characteristic maxima in both acids and alkalies, as found by these workers, correspond to the maxima of the alcohol curves as found by the writer. Ostwald, however, finds in addition an initial minimum (in swelling) which is more pronounced in acids but discernible in alkalies also. In spite of the overwhelming evidence to the contrary of Pauli and his pupils, he attempts to homologize this minimum with

[3] Pauli, W., and Handovsky, H., *Biochem. Z.*, 1909, xviii, 340.

[4] Schorr, C., *Biochem. Z.*, 1911, xxxvii, 424.

[5] Pauli, W., *Z. Chem. u. Ind. Kolloide*, 1908, iii, 2; 1910, vii, 241; 1913, xii, 222; *Tr. Faraday Soc.*, 1913, ix, 54. Pauli, W., and Wagner, R., *Biochem. Z.*, 1910, xxvii, 296. Pauli, W., and Falek, O., *ibid.*, 1912, xlvii, 269.

[6] Lacqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.

[7] Zoja, L., *Z. Chem. u. Ind. Kolloide*, 1908, iii, 249.

[8] Ostwald, W., *Arch. ges. Physiol.*, 1905, cviii, 563; cix, 277; 1906, cxi, 581; *Tr. Faraday Soc.*, 1913, ix, 34.

[9] Procter, H. R., *Kolloidchem. Beihefte*, 1910–11, ii, 243. Procter, H. R., and Wilson, J. A., *J. Am. Leather Chem. Assn.*, 1916, xi, 261. Procter, *J. Chem. Soc.*, 1914, cv, 313. Procter and Wilson, *ibid.*, 1916, cix, 307.

[10] Fischer, M. H., Œdema and Nephritis, New York, 2nd edition, 1915.

[11] Ehrenberg, R., *Biochem. Z.*, 1913, liii, 356.

von Schroeder's maximum viscosity. It is difficult to understand this unless Ostwald has confused the "hydration viscosity" as measured by von Schroeder in dilute solutions with the "gelation viscosity" of solutions which are strong enough to set. The gelation viscosity is of course at a maximum in an approximately neutral solution. It is evident that Ostwald's minimum must be some secondary phenomenon inherent in the swelling method.

The equilibrium between proteins and acids has been studied in more detail by other methods. Bugarsky and Liebermann[12] and Manabe and Matula[13] have made simulfaneous electrometric determinations of the H and Cl ions combined with the protein in HCl. The amount of HCl combined with the protein has been determined by Van Slyke[14] by conductivity measurements, and the amount of the H ion combined has been determined by Pauli and Hirschfeld[15] and by Procter[9] by potentiometer measurements of the H ion concentration. The latter has made perhaps the most notable contribution to the subject by giving a quantitative explanation of the maximum swelling of gelatin in acids.

b. Salts.

The following conclusions can be drawn concerning the effects of salts on the alcohol number of gelatin. (1) All salts increase the alcohol number of gelatin except certain ones which combine a bivalent or trivalent cation with a bivalent or trivalent anion. (2) Trivalent anions and cations are more effective than bivalent, and bivalent are more effective than monovalent in hindering precipitation. (3) The lyotropic effect is of minor importance except in high concentrations of sulfates, citrates, and tartrates.

The results of the experiments with salts alone are plotted in Figs. 2 to 5 and the data are given in the corresponding tables. In all cases the ordinates represent the number of cc. of alcohol required to produce a precipitate in 5 cc. of the gelatin-salt mix-

[12] Bugarsky, S., and Liebermann, L., *Arch. ges. Physiol.*, 1898, lxxii, 51.

[13] Manabe, K., and Matula, J., *Biochem. Z.*, 1913, lii, 369.

[14] Van Slyke, L. L., and Van Slyke, D. D., *Am. Chem. J.*, 1907, xxxviii, 383.

[15] Pauli, W., and Hirschfeld, M., *Biochem. Z.*, 1914, lxii, 245.

TABLE II.

Effect of Various Chlorides (and Other Salts) on the Precipitation of Gelatin by Alcohol.

NaCl		$CuCl_2$		$AlCl_3$		$CaCl_2$		$CuSO_4$		$MgCl_2$		$MnSO_4$	
Concentration of NaCl.	Alcohol to precipitate.	Concentration of $CuCl_2$.	Alcohol to precipitate.	Concentration of $AlCl_3$.	Alcohol to precipitate.	Concentration of $CaCl_2$.	Alcohol to precipitate.	Concentration of $CuSO_4$.	Alcohol to precipitate.	Concentration of $MgCl_2$.	Alcohol to precipitate.	Concentration of $MnSO_4$.	Alcohol to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.	M	cc.	M	cc.	M	cc.
2.0	13.5	1.0	13.4	0.0022	No ppt.	0.52	14.45	0.665	3.5	0.48	13.55	0.5	3.6
1.0	12.2	0.5	13.5	0.0011	6.85	0.26	14.45	0.333	3.6	0.24	12.0	0.25	4.1
0.5	10.85	0.25	14.8	0.00556	5.55	0.13	14.8	0.167	3.35	0.12	10.5	0.125	4.15
0.25	9.45	0.125	19.35	0.000278	4.95	0.065	14.55	0.083	3.05	0.06	10.2	0.0625	4.25
0.125	8.4	0.0625	No ppt.	0.000139	4.65	0.0327	15.1	0.042	3.25	0.03	9.0	0.0312	4.3
0.0625	7.47	0.0312	" "	0.0	4.4	0.0164	12.65	0.021	3.6	0.015	8.05	0.0156	4.1
0.0312	6.6	0.0156	" "			0.0082	10.1	0.0105	4.05	0.0075	7.0	0.0078	4.4
0.0156	6.05	0.0078	" "	Temperature about 30°C.		0.0041	7.8	0.005	4.45	0.00375	6.2	0.0039	4.2
0.0078	5.85	0.0039	" "			0.002	6.5	0.0025	4.7	0.00138	5.4	0.0	4.3
0.0039	5.45	0.00195	7.35			0.001	5.75	0.0013	4.6	0.00069	4.9	Temperature 30°C.	
0.00195	5.3	0.0	4.75			0.0	5.1	0.0	4.6	0.00034	4.75		
0.0	5.1					Temperature 30°C.		Temperature 31°C.		0.0	4.5		
Temperature 30°C.		Temperature about 31°C.								Temperature 31°C.			

For each salt the first column shows the molecular concentration present in the gelatin before the addition of alcohol. The second column shows the number of cc. of 95 per cent alcohol which was added to 5 cc. of the gelatin-salt mixture in order to produce an opaque precipitate; *i.e.*, the alcohol number. Two determinations were made for each point and the average is given in the tables. The concentration of the gelatin before titration was 2 per cent.

ture, and the abscissæ the concentration of salt in the gelatin before titration with alcohol.

In general, all the curves show that with increasing concentra-

TABLE III.

Effect of Various Salts on the Precipitation of Gelatin by Alcohol.

$MgCl_2$.		$CaCl_2$		$MnCl_2$		$Mg(NO_3)_2$		$SrCl_2$	
Concentration of $MgCl_2$.	Alcohol to precipitate.	Concentration of $CaCl_2$.	Alcohol to precipitate.	Concentration of $MnCl_2$.	Alcohol to precipitate.	Concentration of $MgNO_3$.	Alcohol to precipitate.	Concentration of $SrCl_2$.	Alcohol to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.	M	cc.
0.5	11.1	2.85	11.85	0.5	11.25	1.0	13.05	1.63	10.9
0.25	10.95	1.42	11.65	0.25	10.75	0.5	11.9	0.82	11.25
0.125	10.35	0.71	11.5	0.125	10.2	0.25	10.65	0.41	11.3
0.0625	9.9	0.35	11.4	0.0625	9.75	0.125	9.65	0.2	11.2
0.0312	9.2	0.18	11.1	0.0312	9.0	0.0625	8.4	0.1	10.4
0.0156	7.8	0.09	10.1	0.0156	7.5	0.0312	7.35	0.05	9.25
0.0	4.2	0.0							

NH_4Cl		NH_4SCN		$MgSO_4$		$ZnSO_4$*	
Concentration of NH_4Cl.	Alcohol to precipitate.	Concentration of NH_4SCN.	Alcohol to precipitate.	Concentration of $MgSO_4$.	Alcohol to precipitate.	Concentration of $ZNSO_4$.	Alcohol to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.
2.87	12.95	2.0	13.35	0.5	4.4	0.125	2.7
1.43	11.5	1.0	11.35	0.25	4.75	0.0625	3.0
0.72	10.15	0.5	9.75	0.125	5.05	0.0312	3.05
0.36	8.75	0.25	8.4	0.0625	5.25	0.0156	3.1
0.18	7.6	0.125	7.15	0.0312	5.05	0.0078	3.4
0.09	6.65	0.0625	6.35	0.0156	5.15	0.0039	3.85
0.0	4.4			0.0078	4.9	0.00195	4.0
				0.0	4.5	0.0	4.0

* 2 per cent gelatin A, over night at 30°C. Not strictly comparable to the other experiments in this table.

For all except $ZnSO_4$ (as noted) gelatin B was used (3 per cent) and the tubes stood over night at 26°C. before titrating. For further details see Table II.

tions of the salts, more and more alcohol is needed to precipitate until a maximum is reached. Fig. 2 shows that as the valence of the cation increases, the salt concentration at which the maximum is reached decreases. Valence, however, is not the only

factor, $CuCl_2$ being more effective than $CaCl_2$, and $MgCl_2$ less effective. The difference between $CaCl_2$ and $MgCl_2$ is largely due to the fact that acid increases the effect of $CaCl_2$ more than that of $MgCl_2$ and the gelatin used is slighty acid. Similar experiments performed with less acid gelatin show less difference (compare $CaCl_2$ curves in Figs. 2 and 5).

TABLE IV.

Effect of Sodium Salts and Aluminum Sulfate on the Precipitation of Gelatin by Alcohol.

Concentration of salt.	Alcohol to precipitate in		
	NaCl	$NaNO_3$	Na acetate.
M	cc.	cc.	cc.
2.0	11.05	10.5	13.3
1.0	10.1	9.95	10.7
0.5	9.0	9.0	9.05
0.25	8.0	8.0	7.9
0.125	6.85	7.05	6.9
0.0625	6.15	6.05	6.2
0.0	4.4		

3 per cent gelatin B for 15 hrs. at 26°C.

Concentration of salt.	Alcohol to precipitate in	
	Na_2SO_4	Sodium citrate.
M	cc.	cc.
0.5	3.1	4.4
0.25	4.55	6.4
0.125	7.1	8.6
0.0625	8.25	9.45
0.0312	8.15	10.05
0.0156	7.65	10.75
0.0078	5.8	8.95
0.0039	5.1	5.5
0.00195	4.3	4.45
0.00097		4.3
0.0	3.9	3.9

3 per cent gelatin B 15 hrs. at 26°C.

$Al_2(SO_4)_3$.	
Concentration of $Al_2(SO_4)_3$.	Alcohol to precipitate.
M	cc.
0.32	Ppt. without alcohol.
0.23	4.85
0.16	5.3
0.08	5.2
0.04	4.95
0.02	4.89
0.01	4.75
0.005	5.4
0.0025	5.2
0.0012	4.8
0.0	4.6

3 per cent gelatin B for 5 hrs. at 26°C.

Na_2SO_4 and to some extent sodium citrate salt out the gelatin before the true end-point is reached. The white stringy precipitate due to salting out becomes so opaque that in these curves it is taken as the end-point. In $(NH_4)_2SO_4$ in Table V it is possible to titrate through to the usual white homogeneous precipitate. For other details see Table II.

It is also evident from the curves in Fig. 2 that $MnSO_4$ and $CuSO_4$, which combine bivalent cations with a bivalent anion, do not increase the alcohol number at all, but decrease it. This cannot be due entirely to the fact, which Nernst[16] has men-

[16] Nernst, W., Theoretical Chemistry from the Standpoint of Avogadro's Rule and Thermodynamics, London, 3rd edition, 1911, 510.

TABLE V.

Effect of Various Monovalent Salts and Salts with One Bivalent Ion on the Precipitation of Gelatin by Alcohol.

KCl		KSCN		LiCl		$(NH_4)_2SO_4$		(NH_4) tartrate.		$CaCl_2$	
Concentration of KCl.	Alcohol to precipitate.	Concentration of KSCN.	Alcohol to precipitate.	Concentration of LiCl.	Alcohol to precipitate.	Concentration of $(NH_4)_2SO_4$.	Alcohol to precipitate.	Concentration of NH_4 tartrate.	Alcohol to precipitate.	Concentration of $CaCl_2$.	Alcohol to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.	M	cc.	M	cc.
0.5	8.65	1.5	11.6	6.65	16.3	1.0	Ppt. without	0.7	Ppt. without	0.5	11.5
0.25	7.45	0.75	10.5	3.22	13.2	0.5	alcohol.	0.55	alcohol.	0.25	11.4
0.125	6.3	0.375	8.9	1.66	11.4	0.25	9.1	0.39	Sets solid.	0.125	11.1
0.0625	5.65	0.19	7.8	0.83	10.1	0.125	9.5 (6)*	0.19	7.4	0.0625	10.1
0.0312	5.05	0.095	6.7	0.415	8.7	0.0625	9.3 (7)	0.09	9.4	0.0312	9.6
0.0156	4.5	0.046	6.0	0.207	7.7	0.0312	9.1	0.045	9.7	0.0156	7.6
0.0078	4.3	0.023	5.4	0.1	6.6	0.0156	8.0	0.0225	9.4	0.0078	5.7
0.0039	4.0	0.012	5.0	0.05	6.1	0.0078	6.25	0.011	9.2	0.0039	5.0
0.0	3.8	0.006	5.0	0.025	5.5	0.0039	5.3	0.0056	9.1	0.00195	4.3
		0.0	4.7	0.012	5.1	0.00195	4.4	0.0028	8.3	0.00097	4.1
				0.006	4.8	0.00097	4.1	0.0014	6.4	0.0	3.9
				0.0	4.4	0.0	4.0	0.0	5.3		
							3.6		4.3		

* Figures in parentheses indicate approximately the point where the "salting out" precipitate appears (see Table IV).

For all experiments in Table V, 3 per cent gelatin B was used and the tubes stood over night at 26°C. before titrating with alcohol.

The difference between the $CaCl_2$ curve given in this table and that given in Table II is due to the greater acidity of the gelatin A used for the latter. For other details see Table II.

tioned, that such salts are comparatively little dissociated. If the degree of dissociation were the sole explanation, one would expect $CuSO_4$ to have less effect than $CuCl_2$, but not the opposite effect. In some cases such salts do cause a slight increase in the alcohol number in low concentrations, as for example in the curve for $MgSO_4$ in Fig. 3 and for $Al_2(SO_4)_3$ in Fig. 4. $FeSO_4$

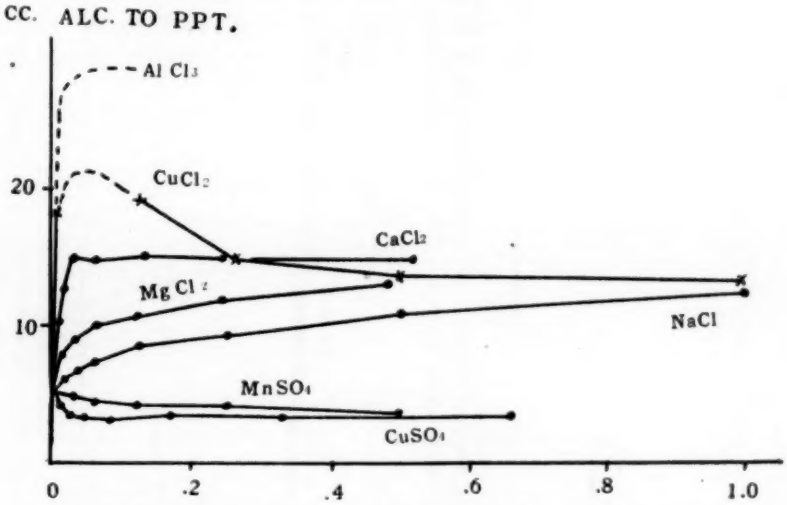

FIG. 2. The ordinates represent the number of cc. of alcohol required to produce an opaque precipitate in 5 cc. of the salt-gelatin mixture; *i.e.*, the alcohol numbers. The abscissæ represent the concentrations of the various salts in the gelatin before titration with alcohol. The effect of the salts increases with the valence of the cation. The dotted lines indicate that no precipitate could be obtained in those concentrations of $AlCl_3$ and $CuCl_2$. Qualitative tests indicate that the $AlCl_3$ curve subsequently comes down a little, like the $CuCl_2$ curve.

also shows a slight increase in the alcohol number in low concentrations.

As proof of the fact that the alcohol number gives a true indication of the effect of salts upon the physical properties of gelatin (in spite of the possibility of repression of ionization of the salts by the alcohol or other interaction) may be mentioned the fact that gelatin sets more readily in solutions of such salts as $MnSO_4$, etc., than in water or solutions of salts with one or

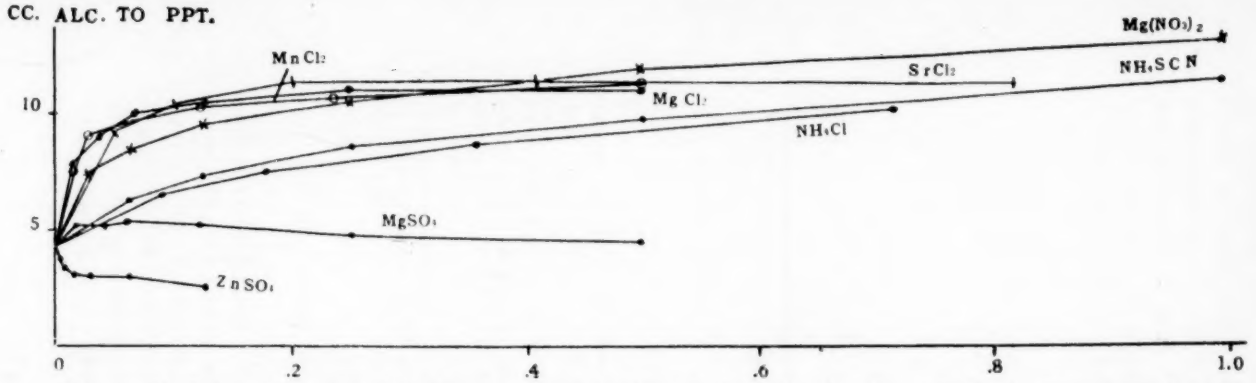

FIG. 3. Alcohol numbers are plotted against concentrations of salts before titration as in Fig. 2. The curves show how clearly three groups of salts are distinguished; *i.e.*, the monovalent salts, salts with one bivalent ion, and salts like $MgSO_4$.

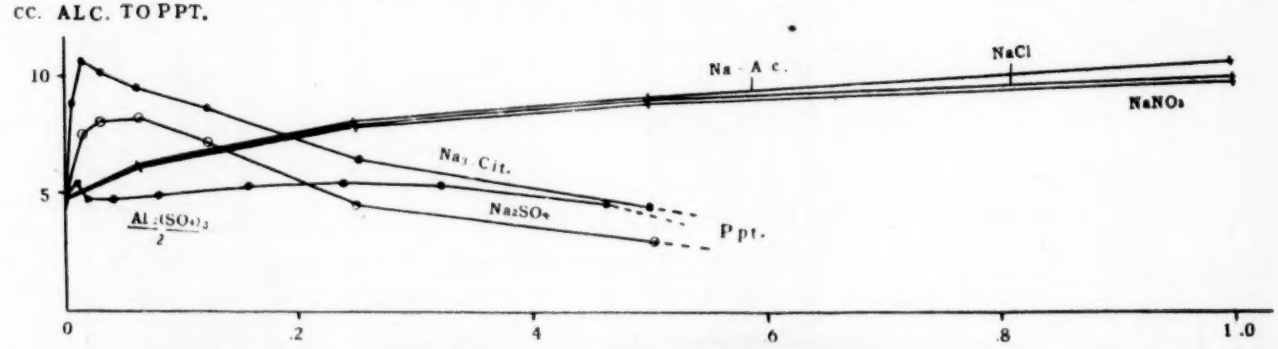

FIG. 4. Alcohol numbers are plotted against concentrations of added salts as in Fig. 2. Sodium citrate has more effect than Na_2SO_4 because it is trivalent or because it has an alkaline reaction, or both. Both these salts "salt out" the gelatin at a concentration of 0.5 M. This accounts for the drop of the curves after the maximum.

two monovalent ions. This indicates that one ion of $MnSO_4$ inhibits the dispersing effect of the other on gelatin.

An examination of the curves for Na_2SO_4, $(NH_4)_2SO_4$, ammonium tartrate, and sodium citrate in Figs. 4 and 5 shows that the valence of the anion is also important but that in high concentrations the "salting out" effect of these salts is predominant. The sharp drop of these curves to the point where they precipitate the gelatin without alcohol is due to their strong dehydrating powers. In the case of these salts there are two dis-

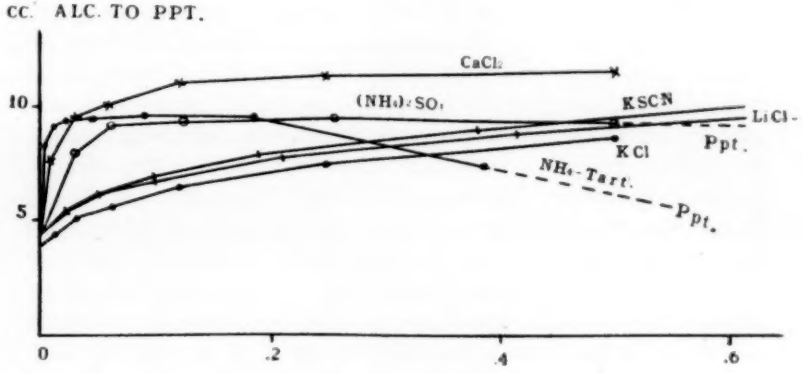

FIG. 5. Alcohol numbers are plotted against concentrations of the salts added to the gelatin as in Fig. 2. This figure shows how distinctly salts with one bivalent ion are distinguished from the monovalent salts in their dispersing action.

tinct end-points to be found in precipitating with alcohol in the higher concentrations. There appears first a stringy precipitate which is followed later by a white homogeneous one. In the case of Na_2SO_4 the stringy precipitate was so opaque that it had to be taken as the end-point, while in the case of $(NH_4)_2SO_4$ it was possible to titrate through to the second precipitate, which had the more usual appearance. This accounts for the fact that the Na_2SO_4 curve drops after the maximum while the $(NH_4)_2SO_4$ curve does not. The first white stringy precipitate is probably due to the "salting out' or dehydrating effect of the SO_4 ion. The second white homogeneous precipitate is regarded as the true end-point.

effect than Na_2SO_4 because it is trivalent or because it has an alkaline reaction, or both. Both these salts "salt out" the gelatin at a concentration of 0.5 M. This accounts for the drop of the curves after the maximum.

In Fig. 5 the distinctness with which the salts with bivalent ions are separated from the monovalent salts is noteworthy.

The effects of salts upon the alcohol number of gelatin are roughly parallel to their effects on the other properties of gelatin. The temperature of gelation in salt solutions has been measured by Levites[17] and by Pauli and Rona,[18] the swelling of gelatin in salts by Ostwald,[8] Ehrenberg,[11] Pascheles,[19] and Lenk,[20] and its viscosity in salt solutions by von Schroeder.[2] None of these methods of investigation is as simple or as accurate as the alcohol method and the results are correspondingly less complete.

Pauli[21] has investigated the effects of salts upon the temperature of coagulation of egg white and finds them similar to the effects on the coagulation by alcohol. He finds that the bivalent metals are more effective than the monovalent metals in preventing coagulation. The action of salts in dispersing gelatin so that more alcohol is needed for precipitation is also similar to their effects in dispersing globulin. Thus Mellanby[22] has found that the dispersing efficiency of trivalent, bivalent, and monovalent ions were to each other as the squares of their valences, and the efficiency of salts were equal to the sum of the efficiencies of their separate ions. Thus the dispersing efficiencies of NaCl, $CaCl_2$, Na_2SO_4, and $MgSO_4$ are 2, 6, 6, and 8 respectively. This conclusion is borne out by the alcohol experiments except for the fact that salts like $MgSO_4$ have very much less effect on gelatin than NaCl, instead of four times as much.

The effects of salts upon the physical properties of proteins are now known to be accompanied by a combination of the salt with the protein, although Bugarsky and Liebermann[12] were unable to find any evidence of it in the case of NaCl and egg white by their measurements of the freezing point or the electrometric determination of the Cl ion. Hardy[23] has shown by conductivity measurements that NaCl combines with globulin to the

[17] Levites, S. J., *Z. Chem. u. Ind. Kolloide*, 1907–08, ii, 237.
[18] Pauli, W., and Rona, P., *Beitr. chem. Physiol. u. Path.*, 1902, ii, 1
[19] Pascheles, W., *Arch. ges. Physiol.*, 1898, lxxi, 333.
[20] Lenk, E., *Biochem. Z.*, 1916, lxxiii, 15.
[21] Pauli, *Arch. ges. Physiol.*, 1899, lxxviii, 315.
[22] Mellanby, J., *J. Physiol.*, 1905–06, xxxiii, 338.
[23] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

extent of 2 per cent, and the writer has found that the same is true for a 1.87 per cent gelatin solution as the following figures indicate:

Concentration of NaCl.	Conductivity depressed.
0.665 M.	1.51 per cent.
0.299 "	1.44 " "
0.145 "	1.66 " "
0.0725 "	1.65 " "

Similar measurements with $CaCl_2$ and Na_2SO_4 both on gelatin and on Witte's peptone have shown that these salts combine more than NaCl.

Pauli[24] has recently made a detailed study of the equilibrium between $AgNO_3$ and gelatin, ox serum proteins, and caseinogen by means of potentiometer and conductivity measurements. He finds that with increasing concentrations of $AgNO_3$ more and more combines with the protein until a maximum is reached. He believes that both ions combine equally. There is evidence that this is not true for the salts of the alkaline earths. Pauli himself states[25] that addition of Ba salts to proteins increases the H ion concentration of the solution as determined by indicators. If this were true it would indicate that the protein combines to a greater extent with Ba than with its anion. Fraenckel[26] has also observed this fact for Ca salts by measurements with a concentration cell. Hardy,[23] furthermore, states that when $CaCl_2$ precipitates globulin, free acid is formed in the solution as determined by methyl orange. Billitzer[27] has found cathodic convection of proteins with the salts of alkaline earths, indicating the presence of a positive charge due to a greater combination of the protein with the cation than with the anion. As further evidence that salts with bivalent cations can deliver a positive charge to colloidal substances may be mentioned the work of Perrin[28] on endosmosis of various solutions through diaphragms of a great variety of substances and the similar work of Briggs,[29]

[24] Pauli, *Biochem. Z.*, 1917, lxxx, 187.
[25] Pauli, *Beitr. chem. Physiol. u. Path.*, 1904, v, 27.
[26] Fraenckel, P., *Z. exp. Path. u. Therap.*, 1905, i, 439.
[27] Billitzer, J., *Z. physikal. Chem.*, 1905, li, 129.
[28] Perrin, J., *J. chim. phys.*, 1904, ii, 601.
[29] Briggs, T. R., *J. Phys. Chem.*, 1917, xxi, 198.

des Bancels,[30] von Elissafoff,[31] and others; and the work of Bose[32] and Guillaume[33] on the potential developed when a gelatin-coated wire is suddenly twisted in the solution of an electrolyte. Contrary evidence is found, however, in the fact that neither Chick[34] nor Hardy[23] were able to find any convection of globulins (or of gelatin) in solutions of the alkaline earths. Whatever difference there is, therefore, in the extent to which the cations and anions of the alkaline earths combine with proteins must be too small to detect except by the most delicate measurements of the change in the H ion concentration. The difference would of course be still less in the case of the salts of the alkali metals.

SUMMARY.

1. Acids and alkalies hinder the precipitation of gelatin by alcohol. In the case of strong acids and alkalies this effect passes through a sharp maximum and then decreases.

2. Increasing concentrations of salts tend to hinder the precipitation of gelatin by alcohol until a maximum is reached, trivalent ions being more effective than bivalent, and bivalent more effective than monovalent in this respect.

3. Certain salts (like $MnSO_4$) which combine a bivalent cation with a bivalent anion are exceptions to this rule in that they either assist or only very slightly hinder the precipitation of gelatin by alcohol, the effect of one ion being apparently neutralized by that of the other.

4. Salts like $AlCl_3$ and $CuCl_2$ which are very effective in hindering precipitation by alcohol resemble the strong acids in that their effect passes through a maximum and then decreases.

5. The effect of the sulfates, citrates, and tartrates of the alkali metals also decreases in high concentrations due to their strong "salting out" or dehydrating powers.

[30] des Bancels, J. L., *Compt. rend. Acad.*, 1909, cxlix, 316.
[31] von Elissafoff, G., *Z. physikal. Chem.*, 1912, lxxix, 385.
[32] Bose, J. C., *J. phys. théorique et apliquée*, 1902, series 4, i, 481.
[33] Guillaume, E., *Compt. rend. Acad.*, 1908, cxlvii, 53.
[34] Chick, H., *Biochem. J.*, 1913, vii. 318.

FEEDING EXPERIMENTS WITH PEANUTS.

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Although, popularly, the peanut (*Arachis hypogæa*) is classed as a nut, it more properly belongs to the grain or forage crop, the "nut" or fruit being the seed of a legume comparable to the bean or pea seed. Its use as human food has been largely limited to peanut butter and confectionery, and little attempt has been made to include it as a regular article of diet. Considerable emphasis, however, has been placed upon its value as food for stock.[1] It forms one of the best forage crops for pigs in the south, while in Europe peanut meal is a much used feed. The good results obtained with it here have suggested that man might profitably add it to his list of staple foods.

In the investigation rats were fed rations consisting of lard, cornstarch, suitable inorganic material, and either ground roasted peanuts or peanut meal. In some cases 2 per cent of butter fat was substituted for an equivalent amount of lard; while in other cases 5 per cent was substituted. One group was given peanut meal to which lard, cornstarch, and butter fat were added, the mineral content being supplied wholly by the peanut meal.

The value of the proteins of the peanut is shown by the performance of animals (Chart I) fed a ration which furnished 18 per cent of protein obtained from peanuts.[2] The rats which were given an insufficient amount of the fat-soluble food accessory failed to grow normally. The addition of 5 per cent of butter fat stimulated growth, and reproduction followed. When very young rats were given this 5 per cent butter fat ration

[1] Henry, W. A., and Morrison, F. B., Feeds and Feeding, Madison, 16th edition, 1916, 178.

[2] The Spanish peanuts used contained 24 per cent protein ($N \times 6.25$).

(Chart III, Rats 4, 6, 7, 14, 17, and 18) growth was not satisfactory, death resulting in some cases, apparently due to the fact that the ration contained too much fat, for in order to obtain an 18 per cent protein mixture from peanuts, together with 5 per cent of butter fat, it was necessary to feed a ration containing 38.6 per cent of fat. Young animals on a diet which includes such a high percentage of fat do not seem to thrive. When these young animals were given a ration containing less fat, obtained by decreasing the amount of butter fat from 5 to 2 per cent, better growth resulted. Their curves of growth, however, were somewhat below normal. An adult animal which was given the 2 per cent butter fat ration reproduced and two litters were successfully suckled (Chart I, Rat 12). The litters were small (five and six each) and the young remained undersized as long as they were given the 2 per cent butter fat ration. The addition of 5 per cent of butter fat stimulated growth.

In view of the fact that peanuts contain such a high percentage of fat, it was deemed advisable to continue the investigation with peanut meal which contained less fat. Rations consisting of 67.5 gm. of peanut meal,[3] supplying 18 per cent protein, 10 gm. of lard, 5 gm. of butter fat, 11.4 gm. of cornstarch, and 5.09 gm. of suitable inorganic material proved satisfactory in every way. The curves of growth (Chart II) in all cases were similar to those generally considered to be normal. Reproduction occurred at frequent intervals and three generations were obtained. Good growth was secured also with a ration supplying 15 per cent protein from the peanut meal (Chart II). The proteins of peanuts are comparable to those of the soy bean, since it has been shown that both legumes supply the essential amino-acids in sufficient amounts for normal growth and reproduction, when rations made on the basis of 15 and 18 per cent protein are fed.

That peanuts are lacking in the fat-soluble food accessory has been shown by the behavior of animals which were given rations containing no butter fat, 2 per cent, and 5 per cent of butter fat, respectively. The animals which, immediately following the suckling period, were placed on rations, otherwise adequate but

[3] The peanut meal was furnished by the Capital Grain and Feed Co., Montgomery, Ala., and contained 27.02 per cent protein (N × 6.25).

containing no butter fat, grew at about half the normal rate and finally died unless butter fat was given (Chart III). The prompt recovery of the surviving animal (Rat 8) when 5 per cent of butter fat was added indicates that the lack of the fat-soluble food accessory was the inhibiting factor in this diet. The animals receiving the 2 per cent butter fat ration made somewhat better growth than those receiving none. Their curves of growth, however, are below those of normal animals (Chart II). Three females of the group reproduced (Chart I, Rats 6, 12, and 13). Their young were also undersized. Irritable dispositions and marked roughness of coats characterized all the animals of this group. Such conditions indicate malnutrition. The change from the 2 per cent butter fat ration to a 5 per cent ration stimulated growth, and reproduction quickly followed in the animals which had not previously reproduced (Chart III, Rats 7 and 14).

Since all the animals receiving the 5 per cent butter fat ration were normal in every respect, proof is furnished for the presence of a considerable amount of the water-soluble food accessory in the diets. When the peanuts formed 56 per cent of the ration, there was sufficient water-soluble B for the experimental animals.

A comparison of the inorganic content of the peanut and the soy bean shows the peanut to be even poorer than the soy bean in certain respects; namely, calcium, potassium, magnesium, and sulfur.[4] Therefore since the mineral content of the soy bean had been found lacking,[5] various amounts of suitable minerals were added to make the mineral content of the peanut rations similar to that of milk, which previous investigations have demonstrated to be adequate for normal growth of rats. On the other hand, peanut meal,[6] which includes a considerable proportion of hulls, apparently contains sufficient amounts of these necessary inorganic constituents for physiologic well being. Rats fed a mixture of peanut meal furnishing 18 per cent of protein, 5 per cent of butter fat, lard, and cornstarch, to which no minerals were added, grew normally, reproduced, and successfully reared their young (Chart II, Rats 27, 28, and 29).

[4] Forbes, E. B., Beegle, F. M., and Mensching, J. E., *Ohio Agric. Exp. Station, Bull. 255*, 1913.

[5] Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91.

[6] *Alabama Agric. and Ind. Dept., Bull. 76*, 1916.

When we consider the broad areas which may be adopted as fitting habitats of the peanut, and the fact that our food supply tends toward a wider use of the seeds of plants, we do not hesitate to venture the suggestion that the peanut, when rightly supplemented, will form a staple article of the human dietary. Like the soy bean, it needs only to have added suitable inorganic material and the fat-soluble food accessory to make it a complete food.

Ration I.

Peanuts	75.0
Butter	2.0
Starch	16.9
KH_2PO_4	1.5
KCl	1.3
$CaSO_4H_2O$	3.3

Ration II.

Peanuts	75.0
Butter	5.0
Starch	13.9
Inorganic material same as Ration I.	

Ration III.

Peanut meal	67.5
Butter	5.0
Lard	10.0
Starch	11.4
Inorganic material same as Ration I.	

Ration IV.

Peanut meal	56.0
Butter	5.0
Lard	10.0
Starch	22.7
KH_2PO_4	1.7
KCl	1.3
$CaSO_4H_2O$	3.3

Ration V.

Peanut meal	67.5
Butter	5.0
Lard	10.0
Starch	17.5

Ration VI.

Peanuts	75.0
Lard	2.0
Starch	16.9
Inorganic material same as Ration I.	

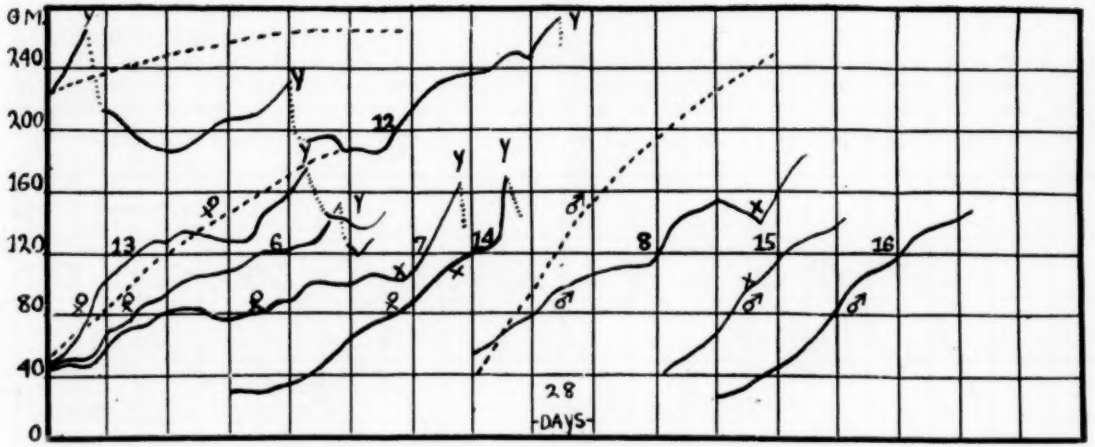

CHART I. Curves of growth of animals fed rations containing 18 per cent of protein, obtained from ground roasted peanuts, a suitable inorganic mixture, and 2 per cent of butter fat (Ration I). Growth was in all cases below normal. Rats 6, 12, and 13 reproduced on this diet and the young, although small, were successfully reared. The addition of 5 per cent of butter fat (Ration II) to the diet of Rats 7, 8, 14, and 15 (indicated by $\times$) stimulated growth. The broken line indicates the normal curve of growth.

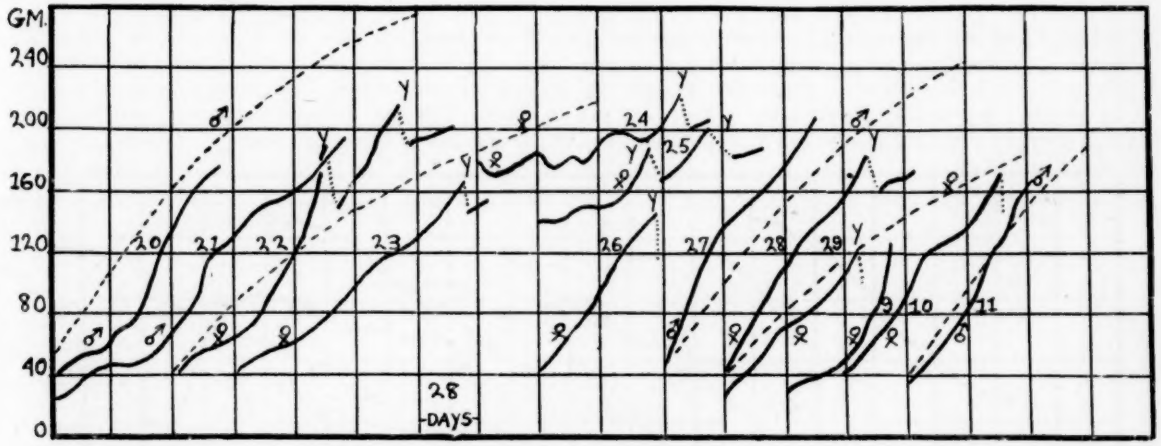

CHART II. The performance of animals (Rats 20 to 26) fed rations containing 18 per cent of protein obtained from peanut meal together with 5 per cent of butter fat and a suitable inorganic mixture (Ration III) demonstrates the efficiency of the proteins of the peanut. Three generations were obtained on this diet. The animals (Rats 9, 10, and 11) on the 15 per cent protein ration (Ration IV) further demonstrate the efficiency of these proteins.

Rats 27, 28, and 29 were given an 18 per cent peanut ration obtained from peanut meal (Ration V), no supplementary inorganic material being included in the diet.

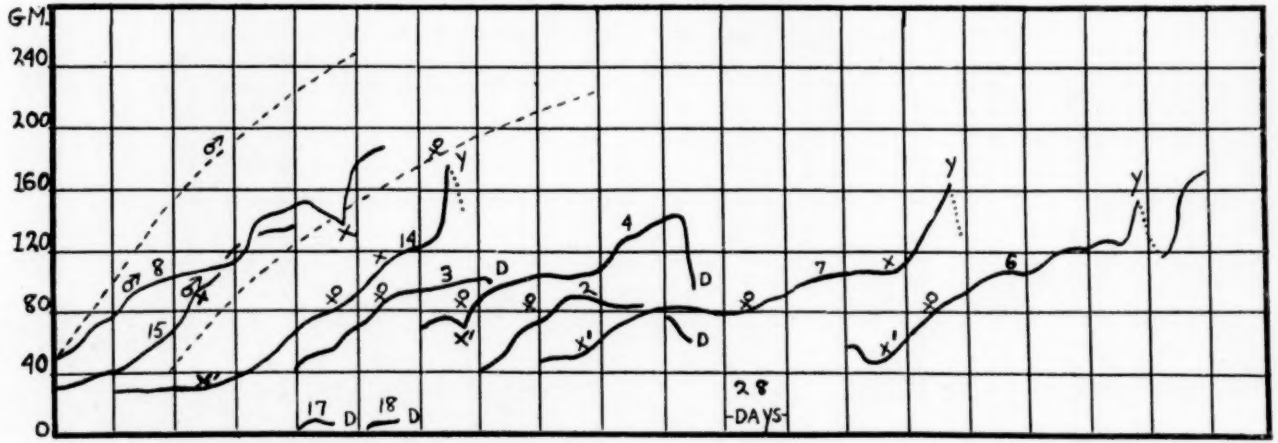

CHART III. Peanuts supply an inadequate amount of the fat-soluble food accessory. These animals (Rats 2 and 3) fed a ration consisting of 18 per cent protein and a suitable salt mixture (Ration VI) grew at about half the normal rate during the 4 months and died. The prompt recovery of Rat 8 when butter fat was added (×) indicates that the fat-soluble accessory was present in too small an amount in this diet. 2 per cent butter fat (Ration I) produced somewhat better growth (Rats 4, 6, 7, 14, and 15). The addition of 5 per cent butter fat to the diet of Rats 7, 14, and 15 stimulated growth.

Very young rats (Nos. 4, 6, 7, 14, 17, and 18) on an 18 per cent protein diet obtained from the peanuts together with 5 per cent butter fat (Ration II) failed to grow. The change to a 2 per cent butter fat ration (×¹) which supplied less fat content resulted in better growth, 5 per cent butter fat (×) added later further stimulated growth.

A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS.

IV. THE CAUSES OF FAILURE OF MIXTURES OF SEEDS TO PROMOTE GROWTH IN YOUNG ANIMALS.

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Our investigations on the composition of the diet in its relation to growth have made it clear that a food mixture may conform to the most approved standards with respect to its energy and protein content and in affording a considerable variety in flavors, and may be so prepared as to be highly attractive in appearance, and still be of such nature as to fail to nourish properly an animal during growth. Such conditions are easily fulfilled if the diet is restricted to dishes prepared wholly from the seeds of plants or their milling products together with starch, sugars, commercial syrups, vegetable fats, lard, flavoring extracts, and spices.

Our extensive studies of seeds in which a single variety was fed with the addition of single or multiple purified food ingredients, have indicated that the seeds all show a close similarity in their dietary properties. Chemical analysis has revealed pronounced differences in the yields of the various amino-acids obtainable from isolated vegetable proteins. Tests by biological methods have established the fact that corresponding to these differences in the constitution of the proteins are equally great variations in nutritive values. In the preceding paper of this series (1) we have shown that the value of the protein mixture present in each of the more important seeds is without exception much lower than are those of milk. Judging from our studies on the physiological minimum of protein for maintenance in the rat, if milk proteins are assigned the value of 100, oat and millet seed proteins would be assigned a numerical value of

about 75, wheat, maize, and rice proteins 50, flaxseed proteins 40, and the proteins of the pea and navy bean about 25. Since the vegetable proteins from various sources exhibit such great variations in their content of the several amino-acids which can be approximately quantitatively estimated, it is reasonable to expect that mixtures of seeds from various sources should in many cases show greatly enhanced values with respect to the biological value of the protein mixture over that of the seeds fed singly as the sole source of protein.

Mixtures of certain seeds should, therefore, on theoretical grounds, be adequate as a source of protein, and the dietary inadequacy of such mixtures should be limited, in the light of our past experience, to a relative shortage of the fat-soluble A and as to the character and amount of the inorganic content. The following table, which was compiled from data by Forbes (2), shows for the seeds, which are of greatest importance as foods in the United States, the great similarity in their content of all inorganic elements except magnesium and calcium. The table also contains data relating to the inorganic content of corn meal and bolted flour.

Mineral Constituents of Seeds. Parts per 100 of Dry Substance.

	Ash.	K	Na	Ca	Mg	P	S	Cl
Wheat	2.08	0.504	0.026	0.041	0.141	0.408	0.009	0.058
Rice, hulled	0.39	0.070	0.016	0.009	0.026	0.091	0.001	0.0004
Oats, rolled	1.83	0.036	0.058	0.097	0.086	0.385	0.005	0.098
Corn	1.55	0.320	0.052	0.011	0.125	0.336	0.160	0.045
Soy beans	3.14	1.162	0.023	0.119	0.169	0.503	0.034	
White beans	3.22	1.177	0.036	0.154	0.148	0.500	0.052	
Cottonseed meal	7.48	1.847		0.236	0.686	1.500	0.037	
Linseed meal	5.84	1.179	0.063	0.351	0.558	0.806	0.076	
Peas		0.940	0.072	0.139	0.150	0.370	0.264	0.034
Barley	1.99	0.270	0.061	0.011	0.150	0.285	0.024	
Patent flour	0.51	0.145	0.003	0.025	0.025	0.111		
Corn meal	0.68	0.163	0.018	0.031	0.061	0.134		
Milk (cow)	5.67	1.160	0.344	0.909	0.088	0.650	0.057	0.791
" (human)	2.42	0.679	0.165	0.288	0.031	0.240	0.018	0.445

With the exception of the calcium and magnesium contents the seeds generally resemble each other in most respects in the character of their inorganic content. We have described experiments which show that each of the more important seeds fails to supply an inorganic mixture of a suitable character to support growth (3). The similarity in the inorganic content of the seeds would suggest that mixtures of seeds should be little better with respect to this dietary factor than are the seeds when fed singly. The experiments reported in this paper demonstrate that this is the case.

In the present series of papers in which we are analyzing by biological methods mixtures of foods which are representative of the diets employed in those regions where pellagra is prevalent, we are proceeding from the simple to the complex mixtures determining in each case the exact nature of the dietary deficiency.

The seeds of plants necessarily form a very large part of the diet of man, because when ripe they are sufficiently dry to make them not difficult to keep in a wholesome condition during a long period. Those which are widely employed as food have either when raw or develop during cooking flavors which render them acceptable to the palate. This is in marked contrast to the leaves of plants which with few exceptions contain tannin or other bitter substances which render them unappetizing to man. It was never suspected that the seeds as a class had any common dietary deficiencies until these were made evident by our systematic feeding of each seed with single and multiple additions of the purified food substances, protein, inorganic salts, and the fat-soluble A, in all possible combinations. The biological values of the several food factors contained in the most important seeds, as revealed by this method, have been discussed at length in preceding publications (3).

The fact that the seeds are not complete from the dietetic standpoint has doubtless escaped observation because of the almost universal use of fairly liberal amounts of the flesh of animals and the products of the dairy in this country and in Europe. Moderate amounts of milk supplement the deficiencies of the seeds most satisfactorily and its use forms the greatest factor of safety in our diet.

As yet very little is known regarding the supplementary relationships of the proteins of one seed for those of another. One of us (4) has recorded observations on swine which were fed mixtures of two of the cereal grains, nitrogen balances being kept on the animals during a period of growth. These records did not show any marked increase in the value of these protein mixtures for growth as compared with the proteins of the individual grains fed singly. Since at that time the necessity of adding certain salts and a growth-promoting fat was not appreciated, the data obtained cannot be now regarded as satisfactory, and the subject needs further study.

We have in former papers emphasized the principle that it is possible to assign to a food factor a biological value only when the values of all the other factors in the diet are known and remain constant. Thus an animal may be able to grow at the normal rate on a diet, the proteins of which are of such a quality as to scarcely make possible the retention of the necessary amount of nitrogen to support normal growth, if the inorganic content and the two unidentified factors are very satisfactory. On the same food mixture so modified as to make the mineral content less satisfactory, the same protein supply will fail to nourish the animal properly. Distinct benefit can be shown with such rations to follow the improvement of the protein moiety of the diet, when the salt content remains less satisfactory than the optimum. In other words, if one takes an ideal diet as 100 per cent, one factor in that diet can be lowered on the scale to 60 per cent and the animal may make a nearly normal performance, but if two factors are lowered to the value of 70 per cent each, the animal might make a three-fourths normal growth, but if one factor were lowered to 50 and another to 60, signs of malnutrition soon become apparent. As was pointed out in a previous paper (1), one cannot say what is an adequate supply of a dietary factor unless the *biological value* of each of the others is known.

In the past because of the absence of adequate knowledge concerning the number of factors which are essential in the diet and great paucity of data regarding the differences in the quality of the proteins from different sources, students of dietetics have placed great reliance on the energy content of the food mixture as shown by calorimetric studies and the protein content as shown by

applying the conventional factor to the nitrogen content, as the criteria by which the ration should be judged. Discussions of the economics of diet have therefore not infrequently, even in very recent times, presented as the main thesis the relative cost of 100 calories of energy and the cost of 100 gm. of protein. As a phase of the discussion of the diet in its relation to pellagra, we wish to illustrate clearly how far these data may fail to indicate the value of a food mixture, especially during growth.

Aron (5), in his studies of the dietaries of the Filipinos, states that the diet of many of the poor laborers consists essentially of about 700 gm. of rice and 250 gm. of fish, the energy value of the fish being equivalent to about 60 gm. of rice. Dairy products are not eaten by these people, and Aron states that the energy value of the fruits and fresh vegetables eaten by his subjects was so small that it might be neglected in his records. Beri-beri is very prevalent where such a monotonous and inadequate diet of rice and fish is eaten.

Chittenden and Underhill (6) have recently described a condition in dogs, which corresponds closely to the syndrome of pellagra in man. They produced this condition by feeding the animals a monotonous diet which consisted of crackers, peas, and cottonseed oil—a diet derived wholly from the seeds of plants. Goldberger (7) has described experiments in which he has produced in man what he and other clinicians familiar with the disease believed to be incipient pellagra, by restricting the diet during 5½ months to dishes prepared from corn meal, corn grits, wheat flour, rice, sugar, starch, syrup, pork fat, sweet potatoes, cabbage, collards, turnip greens, and coffee. We have pointed out (8) that the energy content of the diet was derived to the extent of about 95 per cent from seeds and pork fat. It is with the purpose of emphasizing the impossibility of making an adequate diet from seeds or seed products that we present the data in the accompanying charts. The experimental diets described, illustrate in addition, however, the exact nature of the deficiencies of such seed mixtures. They make clear the necessity both in human nutrition and animal production of supplementing the seeds with either milk or the leafy vegetables in liberal amounts if growth in the young is to be satisfactory, and if the vitality as reflected in longevity and the capacity to produce and rear large

numbers of young is to be fostered. Animals, which have been restricted to inadequate and monotonous diets, have in our experience been subject to infections of the lungs and to a condition suggestive of bronchitis, one or both of which constitute the terminal event in the lives of the animals. Xerophthalmia is seen in those animals whose diet lacks an adequate amount of the fat-soluble A and beri-beri when there is a shortage of water-soluble B. Poor condition of the hair, with varying degrees of baldness in some cases, an incrustation of the ears, irritation of the skin, excessive development of warts, especially on the nose and tail, extreme timidity in certain animals brought to a state of malnutrition which in many instances was manifest only in failure to grow, in others by loss of hair and unthrifty appearance, as well as timidity, are common observations in our rat colony, where many types of faulty diets have been employed. Heightened resistance to disease is one of the outstanding features in those groups of our animals whose diets are of such a character as to induce rapid and sustained growth in the young.

Those who select their food solely with a view to economy as respects cost, find in the literature concerning foods the information that the cheapest sources of both energy and protein are the cereal grains, and the legume seeds, peas and beans. Economic conditions are now such that rigid economy is necessary in the purchase of food, especially among city dwellers with small incomes. Unless the public is speedily educated regarding the necessity of employing regularly in the diet either an appropriate amount of milk or of the leafy vegetables, it seems certain that the time is not far distant when dietary errors now becoming common will become a still more important menace to the public health.

The necessity of increasing the content of sodium, chlorine, and calcium in a diet principally derived from seeds before growth can proceed, affords a striking example of the dependence of the growing animal on an appropriately constituted inorganic mixture as a foundation upon which to superimpose a suitable organic ration. The necessity of an adequate supply of those elements which constitute the ash left on incineration of the animal body has been appreciated since the early experiments of Lunin (9) and of Forster (10) with ash-free diets. It

seems evident, however, that the efficiency of the mucosa of the alimentary tract, the kidneys, and skin in maintaining an appropriate relationship among the inorganic elements in the blood and tissues by selective absorption and selective excretion has been greatly overestimated. These are important factors of safety, but are not sensitive enough to protect the physiological well-being of the animal under conditions which it may meet in taking a diet exclusively of seeds.

Previous to our solution of the problem of the successful feeding of diets consisting of purified protein, carbohydrates, fats, inorganic salts, and appropriate additions to furnish the two unidentified dietary factors, the problem of determining what constitutes the optimum inorganic content in the diet of a mammal was not possible of solution, since only with natural foods could diets be prepared which were capable of supporting growth. The natural foods show considerable variation in their content of mineral elements, and complicated greatly the analytical control of the composition of the food. The greatest obstacle to the study of the problem lay, however, in the fact that every natural food product contains qualitatively all the mineral elements necessary for the adequate nutrition of an animal. It is impossible to remove these, without at the same time extracting certain organic constituents which might possibly change the dietary value of the substance being studied.

Since we have now perfected the technique of preparing from salt-free materials the organic portion of the ration so as to be highly satisfactory when suitable salt mixtures, prepared from pure reagents, are added, we have thus made possible the investigation of the problems relating to the needs of the mammal for inorganic elements during growth and reproduction. These problems are now occupying our attention.

In a previous paper we have described experiments which showed that the leaf of the plant is very different from the seed from the dietary standpoint. In contrast with the cereal grains the leafy portion of the plant is very rich in the fat-soluble A as well as in the water-soluble B, and is likewise much richer in sodium, chlorine, and calcium than are seeds. These differences in dietary properties go with differences in function. The seed is composed of relatively few functioning cells and much

reserve food material. The endosperm is comparable in great measure with a mixture of purified protein, carbohydrate, fats, and inorganic salts. The germ with its high cellular content is relatively richer in both the fat-soluble A and water-soluble B than is the endosperm.

The leaf, on the other hand, is a mosaic of living cells, which is the seat of the great synthetic activities of the growing plant. Corresponding to this difference in function, we have found equally great differences in dietary properties.

SUMMARY OF DATA IN CHARTS.

1. Even with food mixtures derived from two to five seeds we have not found it possible to induce any growth in young rats when the animals were given distilled water to drink, and were therefore, afforded no inorganic salt supply other than what was contained in the seeds. The elements, sodium, chlorine, and calcium, are the only ones which must be added in order to make the seeds complete from the inorganic side. Iodine was given in the drinking water once each week.

2. There is some improvement in the biological values of the proteins of mixtures of seeds in all cases over the values of the proteins of the seeds fed singly. Doubtless in certain cases the values of such mixtures are high, but with simple mixtures of the cereal grains we have not been able to demonstrate a high degree of efficiency as a source of protein in any case.

3. Millet seed and flaxseed are both distinctly better as sources of the fat-soluble A than are the cereal grains, wheat, maize, and oats. Mixtures of seeds containing one of these may, when properly supplemented with calcium, sodium, and chlorine, support animals in apparently good health, and without the appearance of xerophthalmia.

4. Emphasis is laid upon the close similarity of seeds from the dietary standpoint and the danger of deriving the diet almost exclusively from this source.

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2. Forbes, E. B., *Ohio Agric. Exp. Station, Bull. 207*, 1909.

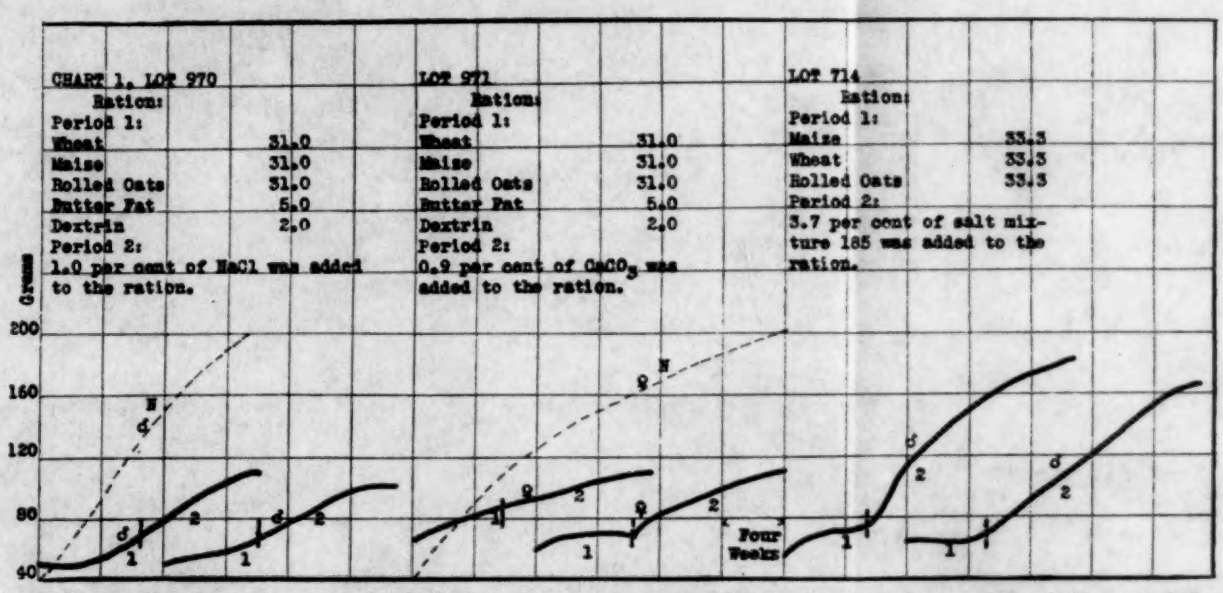
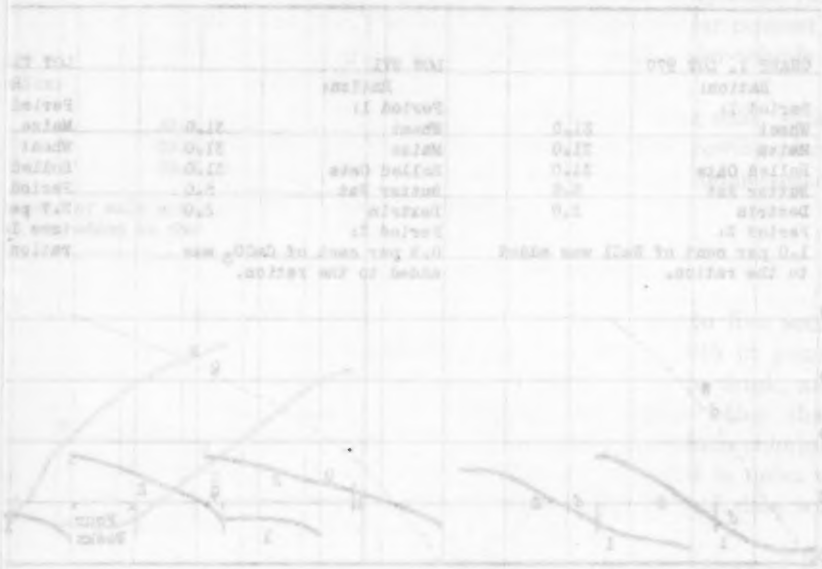


CHART 1. Lot 970. These records show that young rats are unable to grow on a mixture of equal parts of whole wheat, maize, and rolled oats, together with butter fat to supplement the small and inadequate content of fat-soluble A in these seeds. In Period 2, the addition of 1.0 per cent of sodium chloride induced a slight acceleration of growth. That the cause of the slow growth rests in the inadequacy of the inorganic content of the ration, is made evident by the records of Lot 959, Chart 2.

Lot 971. These curves show that the addition of calcium alone (Period 2) to mixtures of wheat, maize, and rolled oats does not permit growth on this seed mixture. The seeds which we have studied are without exception too poor in calcium, sodium, and chlorine, and all must be added before growth can approximate normal and be long sustained (compare Lots 970 and 714, Chart 1, and 959, Chart 2, respectively). The protein content of this mixture of seeds is not of very good quality and the plane of protein intake of animals on this and the closely similar ration described is below the optimum but suffices to support normal growth over a considerable period (compare Lot 714).

Lot 714. These records illustrate that the addition of 3.7 per cent of a complete salt mixture, in Period 2 after 6 weeks of complete suspension of growth, enabled the animals to resume growth at nearly the normal rate on a diet of equal parts of wheat, maize, and rolled oats. The first limiting factor in this mixture is its inorganic content, and sodium, chlorine, and calcium are the only elements which need to be added. We have abundantly demonstrated that these three seeds are too poor in the fat-soluble A to support normal health over a prolonged period. The quality and amount of protein furnished by this seed mixture are below the optimum, but the effects of this would not become manifest except perhaps in failure of the animals to attain the full adult size, or in case the special burden of reproduction is placed upon them.



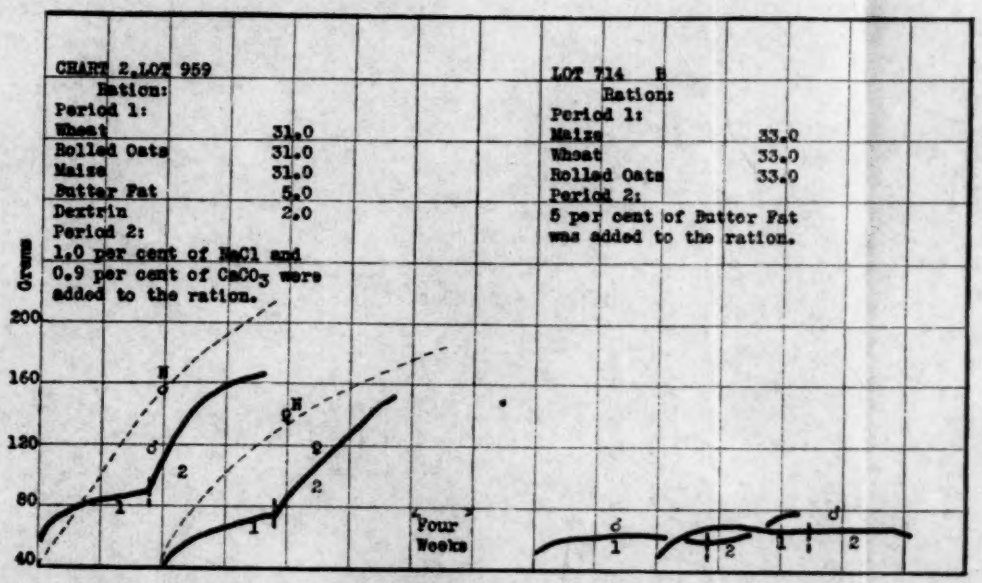
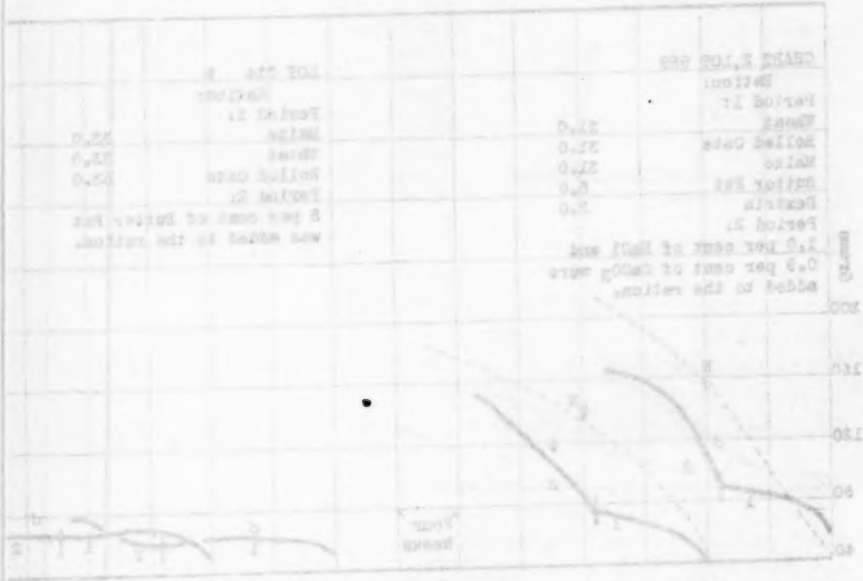


CHART 2. Lot 959. Even after practically complete suspension of growth during 7 weeks, on a mixture of wheat, maize, rolled oats, and butter fat, three of these animals were able to resume growth at once at a rapid rate, when both sodium chloride and calcium carbonate were added to the diet (compare Chart 1, Lots 970, 971, and 714). What is true of this mixture of seeds is true likewise of still more complex mixtures of seeds (compare Lot 715, Chart 6, and Lots 930 and 713, Chart 7).

Lot 714 B. It has been shown that the wheat, maize, and oat kernels are too poor in the fat-soluble A to maintain normal health in animals over long periods (3). Stunting and xerophthalmia being the most prominent sequelæ of this type of specific starvation (11). The addition of a liberal amount of butter fat (fat-soluble A), after a period of stunting, exerts no influence upon the ability of the animals to grow. The first limiting factor is the inorganic content of the food mixture (compare Lots 970, 971, 714, Chart 1, and Lot 959, Chart 2, respectively).



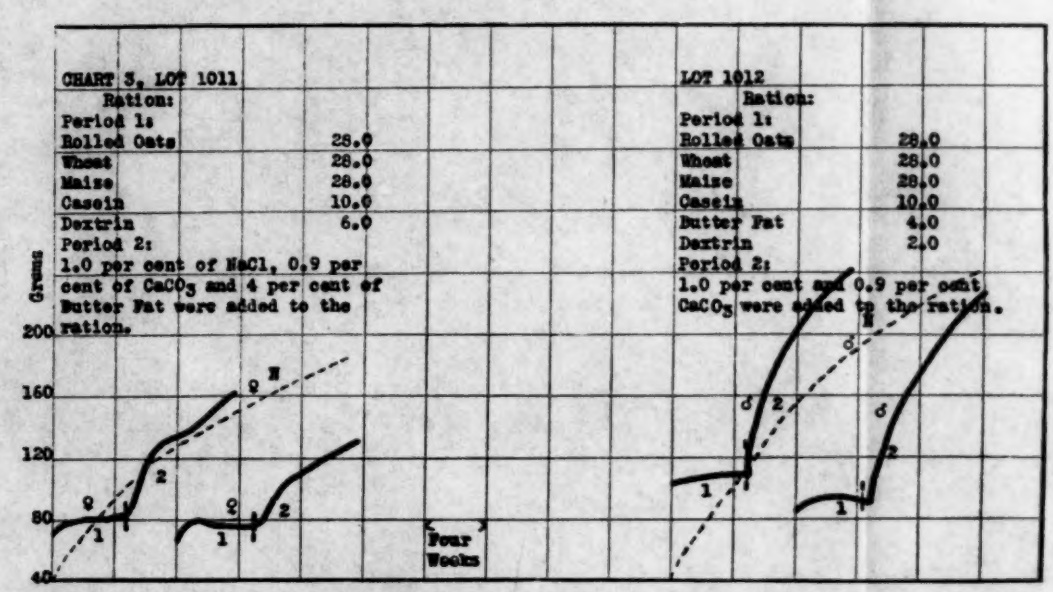


CHART 3. Lot 1,011. These records illustrate the fact that an improvement in the protein content of the diet by the inclusion of 10 per cent of casein, in a mixture of wheat, maize, and oats, does not enable the animals to grow. While it is a matter of importance in determining the vitality of the animals that the protein mixture in this ration be improved and that more of the fat-soluble A be added, the experiments here described emphasize the fundamental importance of having a satisfactory mineral content in the diet.

Lot 1,012. These records are of special interest when compared with the preceding charts. In Period 1, the mixture of wheat, maize, and oats was improved with respect to protein by the addition of casein, and fat-soluble A by the addition of butter fat. Although no growth was possible on this mixture because of the lack of sufficient calcium, sodium, and chlorine, growth was extremely rapid in Period 2 when these salts were added. This result is doubtless due in some measure to the maintenance of vitality in Period 1 of Lot 1,012, better than in Period 1 of Charts 1 and 2, in all of which experiments more than one dietary factor was operating at the same time to depress the vitality of the animals.

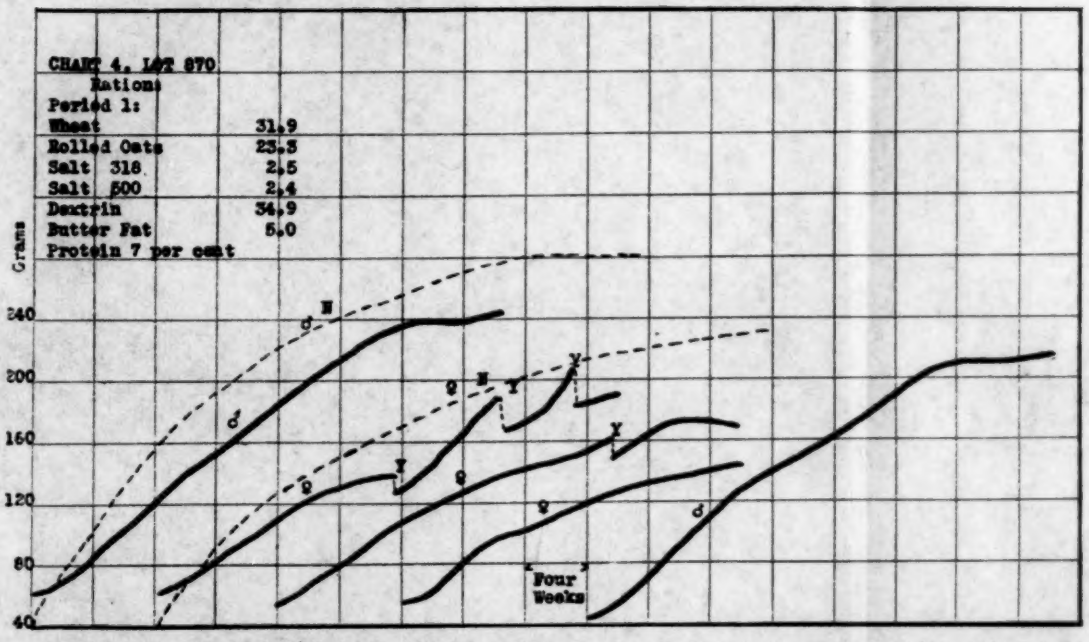
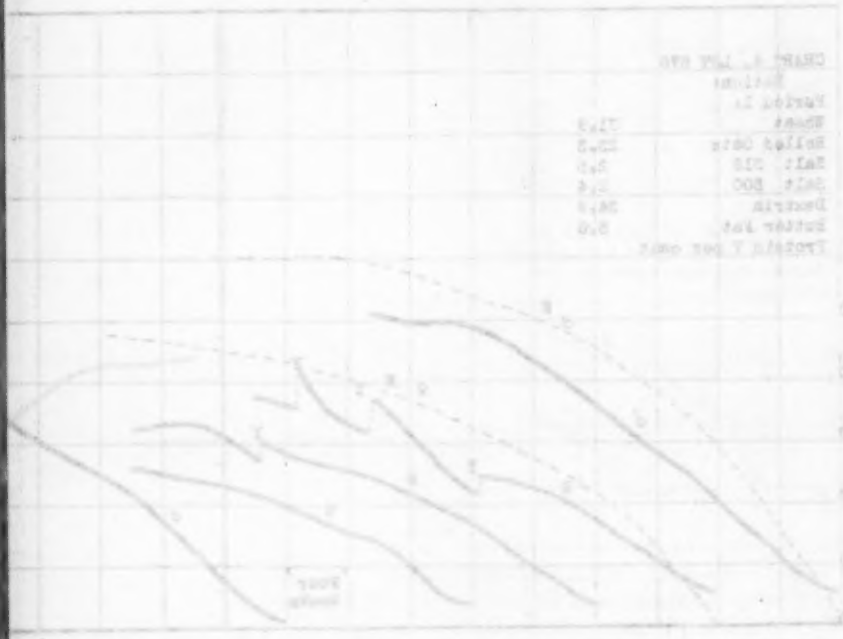


CHART 4. Lot 870. These records show the supplementary relations between the proteins of the wheat and oat kernels. The deficiencies of these seeds were otherwise made good. Under these conditions so low a plane of protein intake as 7 per cent sufficed to induce growth to about 80 per cent of the normal adult size and at somewhat less than the normal rate.

This result is distinctly better than was obtained with 7 per cent of protein derived in equal amount from maize and oats (Chart 5, Lot 869), or from maize and wheat (Chart 5, Lot 868). There is distinct improvement in the quality of these protein mixtures over the biological values of the proteins of each of the seeds when fed separately (1). With rations of the latter type reproduction has never been observed. No young were reared on this ration because of the inability of the mothers to produce sufficient milk on proteins of this quality and in such limited amounts.



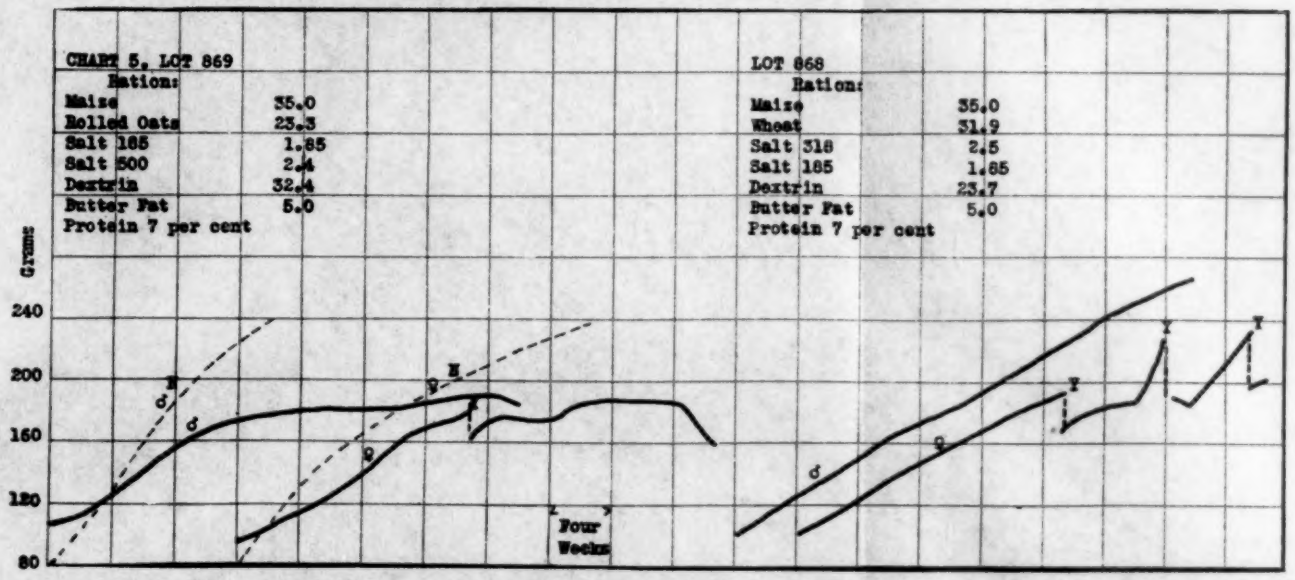
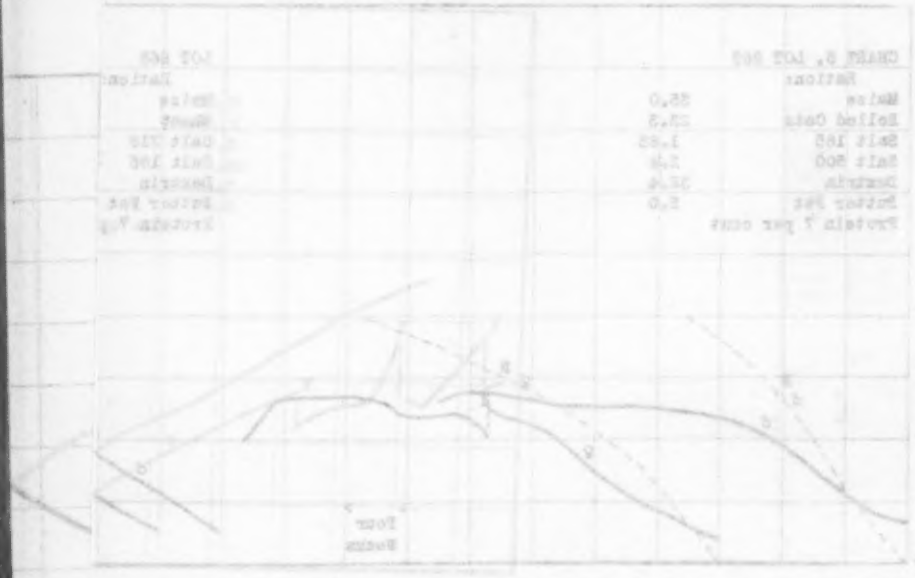


CHART 5. Lot 869. This illustrates the value of the mixture of maize and oat proteins when fed at the plane of intake of 7 per cent of the food mixture. The deficiencies of the grains with respect to inorganic elements and fat-soluble A were made good by suitable additions. The results indicate that this protein mixture is inferior to that derived from the wheat and oat kernels (Chart 4, Lot 870), for growth was not so rapid, and the animals were somewhat more stunted than with the latter ration. The reproduction records on this ration are distinctly below those of Lot 870, on the wheat and oat mixture. Mixtures of maize and oat proteins in equal proportions do not appear to be greatly superior to the proteins of either seed fed singly at the same plane of intake, but some superiority is evident, since no reproduction has been secured on 9 per cent of either maize or oat proteins alone.

Lot 868 shows that wheat and maize proteins when fed together in equal proportions are not of so good a quality as are those of wheat and oats fed together in a similar manner (Chart 4, Lot 870). The fact that the one female produced three litters of young shows clearly that the mixed proteins are better than those of each of the seeds fed singly. These records together with those in Charts 4 and 5 show for mixtures of two cereal grains what we have previously demonstrated for mixtures of maize and navy beans, *viz.*, that the biological values of mixtures of proteins from seeds are distinctly lower than that of the proteins of milk, and emphasize the fact that poor quality of the protein content of the diet is in all probability one of the factors in lowering the vitality of those peoples who live during the winter season on a diet restricted to a few articles, the chief one being corn bread or wheat bread. Corn bread, salt pork, and molasses constitute in winter almost the entire source of nutriment of many people in the South today.



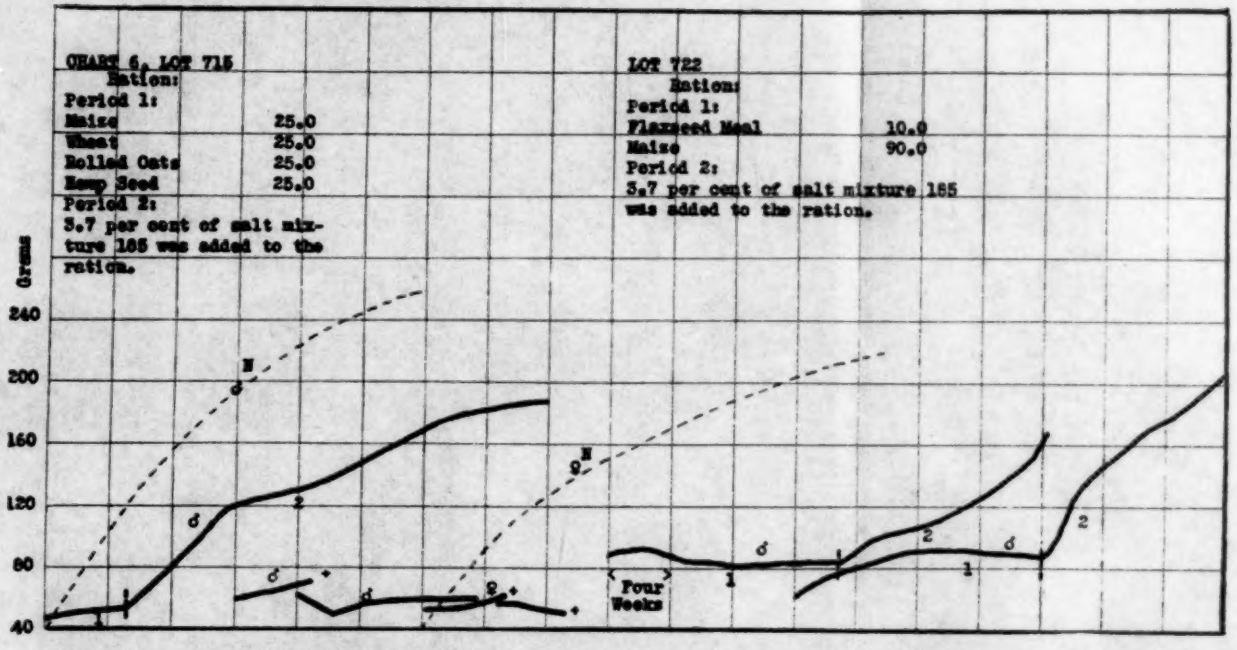
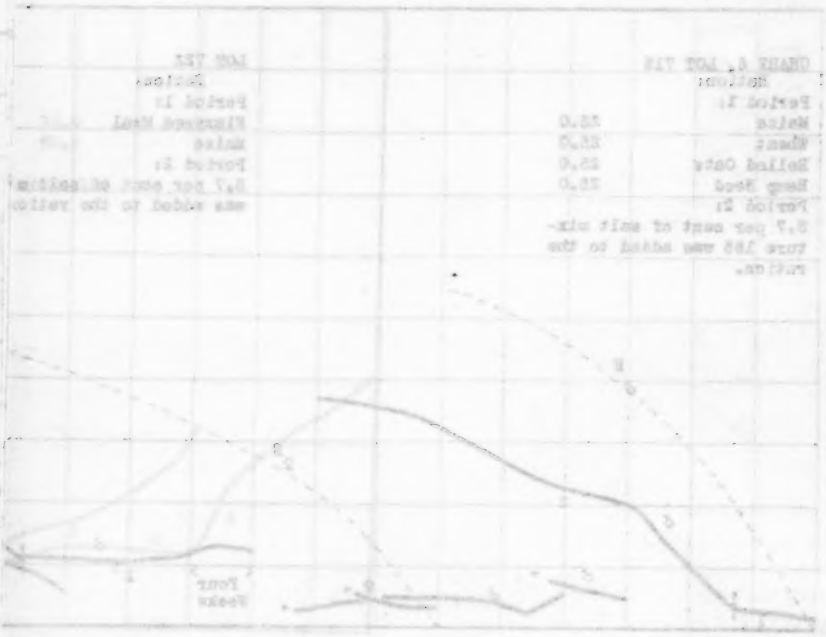


CHART 6. Lot 715. These records illustrate the failure of young rats to grow when fed solely on a mixture of four seeds, maize, wheat, rolled oats, and hemp seed. The prompt response with growth even after long stunting, upon the addition of a suitable salt mixture, adds further support to the belief that the inorganic content is the greatest factor in rendering mixtures of seeds incapable of supporting growth.

Lot 722 shows that growth cannot take place in the young rat on a monotonous food mixture consisting of maize 90 per cent and flaxseed meal 10 per cent. Even after almost complete suspension of growth during 3 to 4 months, there is an immediate response with increase in body weight on the addition of a suitable salt mixture to the diet. The flaxseed differs from the cereal grains in its relative richness in the fat-soluble A. This fact accounts at least in part for the great favor which this substance has found as a stock food.



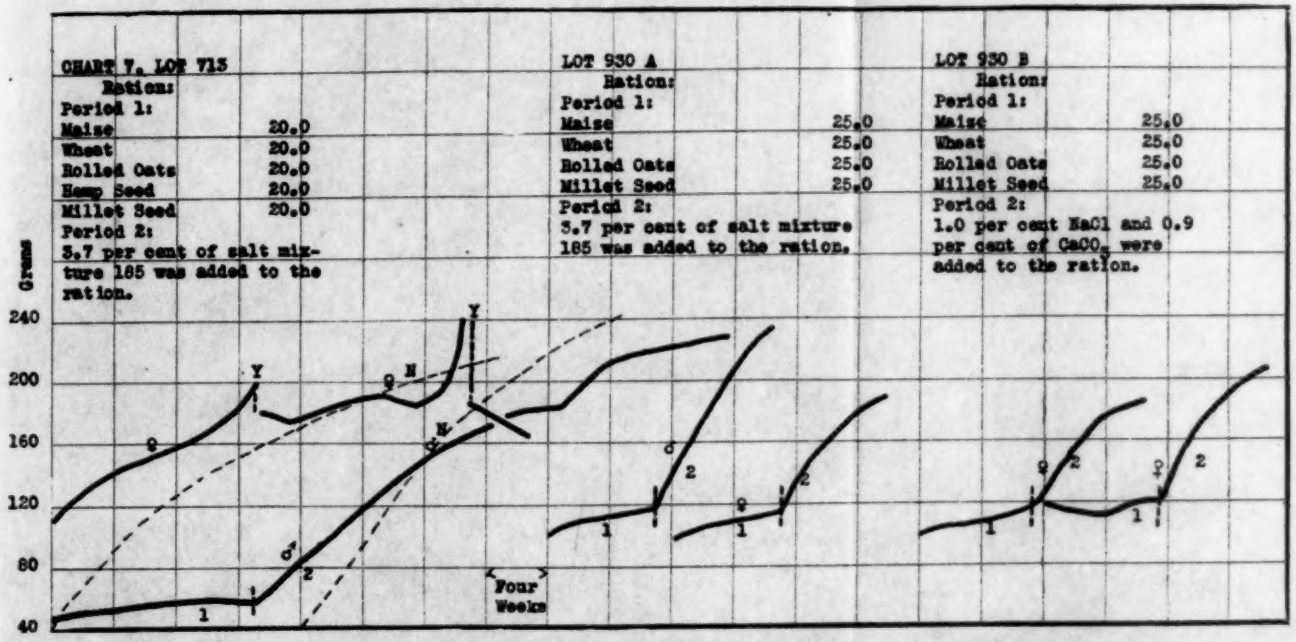


CHART 7. Lots 930 A and 930 B illustrate the impossibility of obtaining growth on a restricted diet of maize, wheat, rolled oats, and millet seed in equal amounts. In Period 2, on the addition of salts, growth at once took place. Lot 930 A was fed a complete salt mixture, while Lot 930 B was given only sodium chloride and calcium carbonate. The response was equally good in each case.

Lot 713 illustrates the failure of young rats to grow when given a monotonous ration of five seeds, and (Period 2) the fact that the limiting factor is the character and amount of the inorganic portion of the diet. Mixtures of seeds of which millet seed is a constituent will be adequate in their content of fat-soluble A provided not less than 20 to 25 per cent of millet seed is present. The diet of the female throughout the experiment was that of the male in Period 2.

Seeds of plants can be classed together without exception in their dietary properties, in that they must be combined with other foods which carry a much greater amount of calcium, sodium, and chlorine in order to render them complete from the dietary standpoint. In lesser degree the poor quality of the proteins of seeds and seed mixtures and the low content (with few exceptions) of the fat-soluble A are contributing factors in causing stunting of animals fed too largely on this class of vegetable foods.

3. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615; xxiii, 181; McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916, xxv, 105; 1916–17, xxviii, 153; 1917, xxix, 341; xxx, 13.
4. McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323.
5. Aron, H., *Philippine J. Sc., B*, 1909, iv, 195.
6. Chittenden, R. H., and Underhill, F. P., *Am. J. Physiol.*, 1917, xliv, 13.
7. Goldberger, J., *J. Am. Med. Assn.*, 1916, lxvi, 471.
8. McCollum and Simmonds, *J. Biol. Chem.*, 1917, xxxii, 29.
9. Lunin, N., *Z. physiol. Chem.*, 1881, v, 31.
10. Forster, J., *Z. Biol.*, 1873, ix, 297.
11. McCollum and Simmonds, *J. Biol. Chem.*, 1917, xxxii, 181.

Salt Mixture 185.

	gm.
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2.H_2O$	0.540
Fe citrate	0.118
Ca lactate	1.30

Salt Mixture 500.

	gm.
NaCl	0.5148
$CaCl_2$	0.2569
K_2HPO_4	0.3113
K citrate	0.5562
Ca lactate	2.878

Salt Mixture 318.

	gm.
NaCl	1.40
K_2HPO_4	2.531
K citrate H_2O	0.710
$CaSO_4$	0.578
Ca lactate	7.058

THYROID HYPERPLASIA AND THE RELATION OF IODINE TO THE HAIRLESS PIG MALADY. I.*

By E. B. HART and H. STEENBOCK.

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PLATES 2 AND 3.

(Received for publication, December 17, 1917.)

In our early experimental inquiries[1] on the growth of swine we soon learned that restriction to any one grain and its protein concentrate or a mixture of grains and seed protein concentrates would lead to nutritional failure. Growth would cease and evidences of serious malnutrition appear. It was found that certain supplements to the grains must be made if physiological soundness was to be maintained and growth continued. Among the efficient supplements were tankage, milk, or the leaf and stem portion of plants, such as alfalfa. The plant roughage or tankage was more efficient than milk, the latter ultimately failing as a sole supplement to the grain for perfect nutrition in the proportion used and under strict confinement of the animals. We believe this failure of milk to rest solely on its quantitative relation to the grain mixture and had enough been supplied, success would have followed. With other species of animals and milk as milk powder constituting 10 per cent of the dry matter of a grain ration, successful nutrition has been attained.

After making clear what classes of materials must be used as supplements to the grains for successful growth,—without as yet having finished the problem as to the complete analysis of the factors for successful nutrition contributed by the supplements,—our animals were involved in reproduction. In a number of cases

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373; *Proc. Am. Soc. Animal Production*, 1915, p. 49; *J. Biol. Chem.*, 1916, xxiv, p. xxviii. Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239.

the young were born dead and hairless or if alive when born hairless, died in a few hours. As it was known to us that this malady existed to a serious extent in certain parts of the northwestern part of this country and also occurred in the state of Wisconsin, our attention was directed to the solution of its cause and remedy. After we were well along in the solution of the problem a paper by Smith[2] appeared from the Montana Agricultural Experiment Station. Smith had reached the conclusion, which our data also support; namely, that the malady was associated with a hyperplasia of the thyroid gland and could be corrected by the use of iodine. He further concluded that the malady was due to a deficiency of iodine in the feed consumed by the brood sows in that locality and believed that if more iodine were fed pregnant animals over large sections of this continent, especially during the winter months, the young that they produced would be more healthy and more vigorous and the large number of weak and defective young animals that are produced annually would be greatly reduced. This last conclusion by Smith is probably open to question, because of the fact that of the vast numbers of farm animals born yearly, but a relatively small proportion are afflicted with those weaknesses that result fatally from a faulty thyroid metabolism and low iodine supply.

Supply of Iodine in Feeds.

The work of Forbes[3] and his associates, and that of Bohn[4] in our own laboratory, show that feeds from all parts of the country are extremely low in their iodine content and with the methods available for its quantitative determination no distinction as to the iodine content of feeds from different localities could be made. It may be a fact that the iodine content of feeds from different regions is very similar, but it is also entirely possible that our analytical methods are still too crude to make the distinctions which may really exist. But while it is true that the iodine content of feeds by the present analytical methods is recorded from

[2] Smith, G. E., *J. Biol. Chem.*, 1917, xxix, 215.

[3] Forbes, E. B., and Beegle, F. M., *Ohio Agric. Exp. Station, Bull. 299*, 1916.

[4] Bohn, R. M., *J. Biol. Chem.*, 1916–17, xxviii, 375.

a trace or none to a percentage in the thousandth place, as for beans 0.0002 per cent, wheat 0.0003 per cent, and rarely reaches a thousandth of a per cent, although corn stover showed 0.0012 per cent and celery 0.0016 per cent, it is equally true that the iodine content of the normal thyroid is relatively low. The normal fresh thyroid of a 300 pound pig will weigh about 50 gm., equivalent to about 7.5 gm. of dry matter with 0.2 per cent of iodine or 15 mg. In 100 pounds of feed with 0.0002 per cent of iodine there are 90 mg. of iodine, or six times that of a normal swine thyroid. The rate of metabolism of the thyroid colloid is unknown. The wonderful avidity of thyroid tissue for iodine, as lately demonstrated by Marine and Feiss and Marine and Rogoff,[5] makes it appear more than probable that it is not, at least in all cases, a deficiency of iodine in the feed *per se*, that is the primary cause of thyroid hyperplasia in swine producing hairless pigs, but that the problem is more complex and related to the absorption of iodine from the digestive tract or by the thyroid gland itself.

We have successfully corrected the malady of hairless pig production by the use of potassium iodide, but would hesitate to prescribe potassium iodide as a necessity for normal reproduction for all regions and all animals. The same feeds that produce hairless and dead pigs can be so combined as to produce normal pigs. What the problem needs is a fundamental study of all the factors that may contribute to the final condition of a deranged thyroid gland and its accompanying faulty metabolism. If certain unknown conditions are favorable a sow may obtain from natural feeds the iodine required for both her own and the fetal thyroids, but if unknown and unfavorable conditions prevail the animal may obtain barely enough for her own thyroid activity, but not enough for fetal thyroid development. We have not completed our studies of the problem, but what information we have accumulated is offered as a preliminary report.

[5] Marine, D., and Feiss, H. O., *J. Pharm. and Exp. Therap.*, 1915, vii, 557. Marine, D., and Rogoff, J. M., *ibid.*, 1916, viii, 439.

EXPERIMENTAL.

After our early experiments on the growth of swine with grains and grain mixtures had demonstrated the incompleteness of such rations and their disturbing influence on reproduction, attempts were made to determine the amount of alfalfa that must be introduced into the ration to secure a complete cycle of life. With 15 per cent of the air-dried ration, as air-dried alfalfa, good growth and reproduction were secured. This particular ration consisted of 25 parts of corn meal, 25 parts of oat meal, 25 parts of wheat middlings, 10 parts of oil meal, and 15 parts of alfalfa. The pigs were strictly confined to the ration in pens 9 by 12 feet, with shavings as litter. They grew well, but their first litters of pigs were born dead and hairless. The hairless pigs were of normal weight and size and were generally carried a little over the full gestation time,—4 to 7 days. If born alive they lived a few hours after birth, but always died sooner or later. They are characterized by thick pulpy necks and thick skin infiltrated with mucin material. The hair coat may vary and appears to bear a particular relation to their vitality. Where hairless, the skin is smooth, shiny and bald, except for a few hairs around the eyes and nose. In the same litter there may be variation from full hair coats with full vigor to the scanty hair coats and little vitality. The hoofs of hairless pigs are thin walled and undeveloped and the thyroid gland is greatly enlarged.

In our experience the fresh weight of the thyroid from hairless new-born pigs may vary from 0.5 to 3.5 gm., while the fresh weight of the thyroid from the normal new-born pig is rarely over 0.3 gm. It is difficult to state just what a normal thyroid is, for there is considerable variation in weight in the thyroids from apparently normal pigs, but in the 50 to 60 thyroids which we have examined from normal 1 day old pigs the fresh weight varied from 0.160 to 0.800 gm.

The thyroid of sows producing normal pigs also varies in size, and sows of 300 pounds' live weight may have fresh thyroids varying in weights of from 20 to 50 gm. The thyroid of sows producing hairless pigs is likewise large and may weigh 125 gm. in the fresh state. In our experience there is not complete involution, through the administration of potassium iodide, of the

thyroid of the mother producing hairless pigs, although it does serve to give the normal sized thyroid to the fetus. As an illustration, a thyroid from a mother that had always produced hairless pigs, but in the last gestation had received potassium iodide, weighed when fresh 112 gm. and was translucent and gelatinous in appearance, indicating a richness in colloid; it contained 0.62 per cent of iodine in the dry matter, which weighed, 33 gm., but the fresh thyroid was of the size of a man's fist while the normal 300 pound pig's thyroid approximates the size of a walnut.

It has already been made clear by the work of Marine[5] and others that the enlarged thyroid, accompanied by the symptoms of myxedema and dermal malnutrition, is a thyroid low in iodine. This enlarged vascular system of the thyroid gland is directly related to an automatic attempt on the part of the organism to furnish a blood of probably lower iodine concentration to the thyroid at a greater rate, in order that this gland may meet the requirements for adequate colloid building. This is exactly the condition of the thyroid in the hairless pig. Its iodine content is either but a trace or none. 1 day old pigs with scanty hair coat contained 0.02 to 0.05 per cent of iodine in the dried thyroid, while the thyroid from the dead and *hairless pigs* contained inestimable quantities of iodine. The iodine content of the *normal* dried thyroid of 1 day old pigs varies from 0.1 to 0.6 per cent. Our own experiments, therefore, confirm in every respect the belief that there is a close relation between the hairless pig malady and the iodine assimilation by either intestine or thyroid of the mother.

After we had produced hairless pigs by the above ration, the same sows were fed during the second gestation period a ration consisting of 33 parts of corn, 33 parts of oats, and 33 parts of alfalfa. On this ration dead and hairless pigs were again produced. With these mothers, once there had been induced serious derangement, through the use of the first ration, of either intestinal absorption or thyroid metabolism, the introduction of a lower protein level and larger amount of roughage did not act as a corrective. Bearing in mind the possibility that alfalfa itself was a contributor to this disturbance the sows were carried through a third gestation on a ration of 33 parts of corn, 33

parts of oats, and 33 parts of clover. Again, hairless and dead pigs were born. In the fourth gestation period potassium iodide at the rate of 10 gm. per 100 pounds of feed was given with the above ration,—33 parts of corn, 33 parts of oats, and 33 parts of clover. On this ration normal, vigorous and healthy offspring were produced. The thyroids from the pigs born in the third gestation were large, weighed 2 to 3 gm. fresh, and contained no iodine. In the fourth gestation, the fresh thyroids of 1 day old pigs weighed from 0.250 to 0.4 gm. and contained 0.3 to 0.4 per cent of iodine in the dry material.

The proportion of potassium iodide fed was arbitrarily chosen and possibly less would adequately correct the trouble. Marine and Kimball[6] say that 1 mg. of iodine given weekly by mouth is ample to prevent goiter in dogs. The rate of 10 gm. per 100 pounds of feed is equivalent to approximately 0.016 per cent of iodine, which is much more than is contained in natural feeds and which can give successful reproduction. If, however, it should be found wise to insure our herds of breeding swine against this trouble, it can be done at the present price of potassium iodide (7 dollars per kilo) for 30 cents per head and used during the full gestation period.

After observing these first results with 15 per cent of alfalfa in the ration and a high protein intake during the growing period, we believed that it would be a simple matter to produce enlarged thyroids and hairless pigs by feeding almost any combination of food materials, particularly from plant sources. Consequently sows of 30 to 50 pounds' weight were started on rations made up of 75 parts of corn and 25 parts of alfalfa; 75 parts of rolled oats and 25 parts of alfalfa; 75 parts of wheat and 25 parts of alfalfa. These animals grew well, although slightly below the normal rate. They were fed natural water as had been the case in all the other experiments, but were strictly confined. The first litters of all of these sows on these rations were strong and vigorous. There was not a scanty hair coat in any of the litters or an enlarged thyroid among those examined. The thyroids weighed from 0.100 to 0.300 gm. fresh and contained 0.25 to 0.40 per cent of iodine in the dry material. The feeds were from the same

[6] Marine, D., and Kimball, O. P., *J. Lab. and Clin. Med.*, 1917, iii, 40.

sources as in the previous experiments and as far as analytical methods could show, contained no more iodine than those used in our first ration.

The difference between the two types of rations was not in the iodine content, but the percentage of protein and the percentage of roughage. The first ration contained 14 to 15 per cent of protein and the second about 10 per cent. The first ration contained but 15 per cent of alfalfa and the second 25 per cent.

It has been believed for some time that the thyroid gland serves as a detoxicating agent and that the hyperplasia results from stimulation to the gland to produce more and more colloid for purposes of detoxication. Such an hypothesis must assume that the iodine compound produced by the thyroid is, under conditions of high toxicity, either eliminated from the blood stream at a more rapid rate than normally, or is insoluble in the blood stream and cannot transfuse the placental wall. It assumes that there is just as much iodine coming into the blood stream through the intestinal walls as under normal conditions. If this is the case, however, it is difficult to comprehend why there should be a deficiency of iodine for the fetal thyroid unless we assume, as stated above, that there is a more rapid elimination of iodine compounds or the formation of iodine compounds insoluble in the blood.

The fact that our first ration was high in protein and low in roughage would suggest an alternate hypothesis. Such a ration would lead to more or less intestinal putrefaction with the accompanying conditions of faulty assimilation. A faulty assimilation of the iodine compounds in the food would lower the iodine supply in the blood. This would lead to the hyperplasia of the thyroid gland of the mother as well as that of the fetus. Or it is possible that the selective absorption of iodine, instead of being wholly referred to the intestine, may be referred directly to the thyroid gland. Toxic substances produced through putrefaction in the intestine or coming directly from the food may, after absorption into the blood, cause a derangement of the iodine-absorbing mechanism of the thyroid gland; only then as the mass of iodine in the blood is further increased by the administration of iodine, can normal absorption by the thyroid gland

be reestablished. This condition could apply to the fetus as well as to the mother. This hypothesis would not preclude the possibility of a goitrous condition due to an actual deficiency of iodine in the food material in certain regions, if it really existed. It would, however, explain the differences observed in the effect of the two types of rations mentioned above and, further, explain the sporadic occurrence of the hairless pig malady. This disease occurs more often in gilts than in old sows. It is more likely to occur in the spring and disappear with the fall litters. On one farm in Wisconsin fifteen from a herd of twenty brood sows produced hairless pigs in the spring of 1917. These sows received a ration of flour middlings, ground oats, skim milk, oil meal, and some ear corn. The slop for the greater part of the winter contained mostly skim milk. This is a high protein ration and probably somewhat constipating. These same sows on summer pasture, with more exercise and roughage farrowed large, healthy litters. The high protein levels fed gilts, the lessened exercise, and more constipating foods of winter are exactly the conditions accompanying the greater prevalence of this malady; and these are the more likely conditions leading to a disturbed assimilation or absorption of iodine.

The hypothesis that the toxicity comes directly from the food materials, as distinct from having its origin in intestinal putrefaction, and leads to a deranged thyroid metabolism is not outside the pale of possibilities. Certain saponins, as digitalin, and toxic substances, as ricin and abrin, are said to produce thyroid hyperplasia. The fact that the hairless pig malady in the northwest is in some cases sharply limited to certain areas, but that within these areas there are unaffected spots, as reported by Smith,[2] strongly suggests the possibility that toxic plants may be concerned in the production of a faulty thyroid metabolism and hairless pigs in some of these localities.

Recently Burget[7] has attempted to produce thyroid hyperplasia in rats experimentally. In this he succeeded through the instrumentality of unsanitary conditions and high protein diets. Further he presents evidence to show that the enlarged thyroid is not produced by specific infection, a view which is in harmony

[7] Burget, G. E., *Am. J. Physiol.*, 1917, xliv, 492.

with our own observations. One of two sows, reared on the same ration and in the same pen, produced hairless pigs and the other produced sound and vigorous offspring. These results can be ascribed to constitutional differences, inherited factors which always display themselves with any group of animals. The ration that is near the border line of deficiency in iodine and at the same time is slightly out of balance in some other respect may well affect some individuals more than others.

The sows that had been reared on grain and alfalfa (75 to 25 parts) with successful reproduction, were next taken to higher protein levels and lower roughage,—a ration of 15 parts of alfalfa, 45 parts of corn, 15 parts of oil meal, and 25 parts of middlings. Here there has also been successful reproduction. The young pigs were well haired, and the thyroids normal and with normal iodine content. It appears probable, therefore, that the disturbances to thyroid metabolism are more likely to be developed during the period of most rapid growth of this species than after maturity, but final conclusions in this respect cannot as yet be made.

SUMMARY.

A brief summary of our experience with the hairless pig malady supports the view that it is occasioned by a low iodine assimilation by either intestine or thyroid, resulting in a goitrous condition in both mother and young. This condition interferes more severely with fetal development than with the normal maintenance of the mother.

It appears to be produced by rations with high protein levels and low laxative effects, with the accompanying condition of lack of exercise and unclean surroundings. Young sows exposed to such conditions are more prone to develop thyroid enlargement than are mature sows. We recognize, of course, that there is the possibility that some rations from certain regions are naturally so low in iodine as to make the scarcity of iodine the direct cause.

We have found it possible to grow sows to maturity on natural feeding materials grown in southern Wisconsin, but of relatively low protein content and good laxative properties, with the production of normal offspring. On the other hand, these same

feeds, combined in different proportions and fortified with protein concentrates lead to the production of hairless pigs.

We are of the opinion, for the present at least, that we have not reached the stage where it is wise to advocate the *general* use of iodine in the feed of all brood sows; that the conditions leading to the production of hairless pigs should first be analyzed fully in order that we may acquire a complete understanding of the reasons for the successful rearing of sows intended for breeding purposes through the use of natural materials, unfortified with potassium iodide.

However, in regions and on farms where hairless pig production is endemic or persistent in character the direct use of iodides should be made.

In the historical, as well as the practical phases of goiter prevention some observations by Marine are of interest. He says:[6]

"The first instance of preventing goiter on a large scale was accidental and in connection with the sheep raising industry of Michigan. Prior to the discovery of salt deposits around the Great Lakes, the future of the industry seemed hopeless, but with the development of the salt industry and its use by the sheep growers, goiter rapidly decreased. The salt contains appreciable quantities of both bromine and iodine. . . . . The second instance of goiter prevention on a large scale was in brook trout. Some years ago the development of goiter in artificially raised members of the salmon family became alarming and many plants were abandoned on account of the disease. After considerable work, which led to the conclusion that the disease was simple goiter, we were able to completely prevent the disease in several hatcheries, by the use of very small amounts of tincture of iodine added to the water."

It is therefore to be expected, that with the elucidation of the cause of the hairless pig malady, a prompt remedy, when necessary, can be applied.

EXPLANATION OF PLATES.

Plate 2.

Fig. 1. Dead and hairless pigs. Note the thick necks, indicating a goitrous condition. The mother of these pigs had been reared on a high protein and low roughage diet, consisting of 15 parts of alfalfa, 25 of corn, 25 of oats, 25 of middlings, and 10 of oil meal, and kept under strict confinement. She had produced two litters of hairless pigs before being changed to a ration of 33 parts of clover, 33 of corn, and 33 of oats, on which the above pigs were produced.

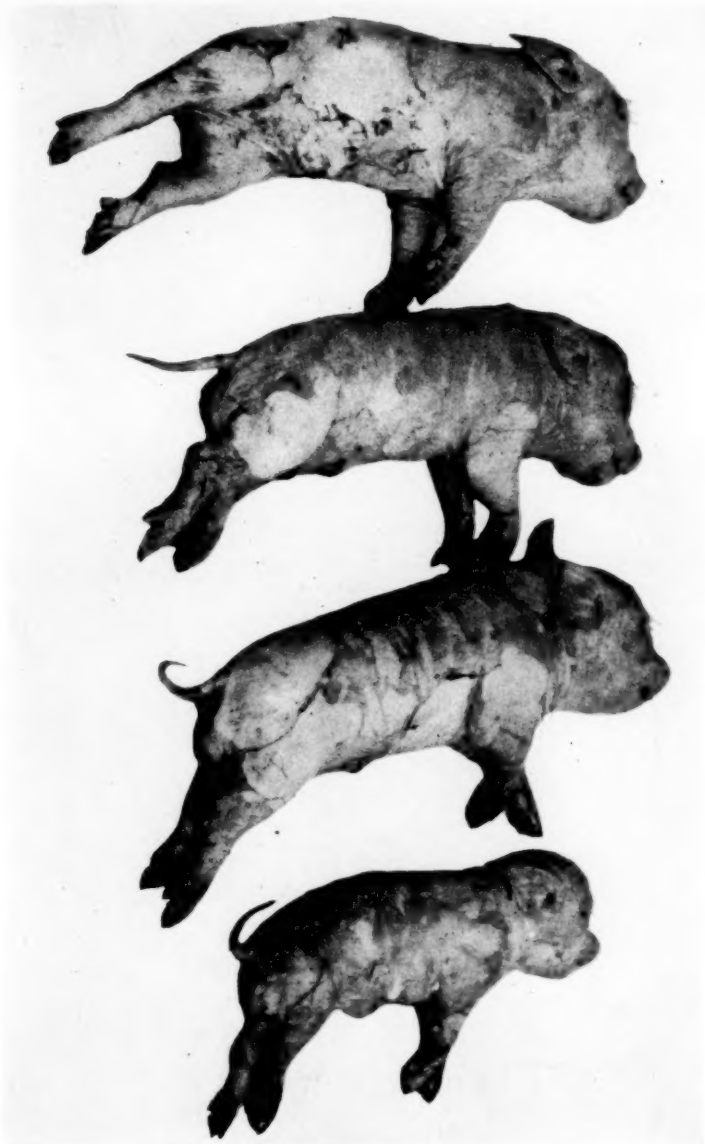

FIG. 1.

(Hart and Steenbock: Hairless pig malady. I.)

FIG. 2.

FIG. 3.

(Hart and Steenbock: Hairless pig malady. I.)

PLATE 3.

FIG. 2. The same sow that produced the pigs shown in Fig. 1. On the same ration,—33 parts of corn, 33 of oats, 33 of clover,—but fortified with 10 gm. of potassium iodide per 100 pounds of feed, a normal litter was produced.

FIG. 3. Reared on a ration of low protein and comparatively high roughage content, 75 parts of corn, 25 of alfalfa. The iodine content of this ration was not unlike that of the ration which produced the results shown in Fig. 1; the two rations, however, differed in their protein and roughage content and in this case the low protein diet was consumed during the life of the animal, including the growing period.

ON THE ESTIMATION OF AMINO-ACID NITROGEN IN THE BLOOD.

By SEIZABURO OKADA.

(From the Medical Clinic, Imperial University of Tokyo.)

(Received for publication, December 13, 1917.)

Since the publication of the nitrous acid method for the determination of amino-acid nitrogen by Van Slyke[1] in 1909, the literature upon the occurrence of amino-acids in blood and tissues has grown rapidly. Various procedures have been proposed by different authors for the preliminary removal of protein necessary to the analysis. For the most part the original method of Van Slyke and Meyer[2] has been followed, in which the blood proteins are removed by precipitation with nine or ten volumes of 95 per cent alcohol.

Recent investigations by Folin and Denis,[3] Greenwald,[4] and Bock,[5] however, have shown that the use of alcohol is not effective owing to the fact that amino-acids do not completely escape precipitation by alcohol. Folin and Denis state that certain nitrogenous substances such as creatine, asparagine, and tyrosine added to the blood could not be recovered quantitatively after precipitation with methyl alcohol. Greenwald found that methyl alcohol filtrates contained only a little over half of the amino-acids added to the blood. After a number of experiments with several protein precipitants, he found that trichloroacetic acid closely approximated the ideal precipitant. Nine volumes of 2.5 per cent solution to one volume of blood were adopted and the filtrates treated with a little kaolin. It was found, in every case, that the trichloroacetic acid filtrate contained all the added amino-acid nitrogen. The liquid remains clear after the addition of picric acid or of potassium mercuric iodide, giving no biuret reaction. Bock, as a result of his study on this question, concluded that the trichloroacetic acid procedure gives satisfactory results,

[1] Van Slyke, D. D., *Proc. Soc. Exp. Biol. and Med.*, 1909–10, vii, 43; *J. Biol. Chem.*, 1911, ix, 185.

[2] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

[3] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

[4] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

[5] Bock, J. C., *J. Biol. Chem.*, 1916–17, xxviii, 357.

but is somewhat troublesome in certain stages of the manipulation. Filtration is very slow, and the alternative process of centrifuging such large volumes (300 to 500 cc.) is not always convenient. To avoid this difficulty, he recommended heat coagulation followed by trichloroacetic acid precipitation.

From the standpoint of accuracy the procedures of Greenwald and Bock are both satisfactory, but some difficulties still remain, chiefly the removal of the trichloroacetic acid after precipitation of protein. As suggested by Bock, if the trichloroacetic acid is not entirely removed it enters into some combination with the amino-acids which prevents their quantitative reaction in the Van Slyke method. Vacuum evaporation does not suffice for this purpose and Bock's procedure of direct evaporation until the color change of the alizarin indicator is not convenient, for, as the solution reaches dryness, frothing and bumping often occur; further, the operation requires from 30 to 50 minutes. After making alkaline to remove ammonia and acidifying again, it is difficult to get clear solutions. To avoid this difficulty, I sought to obtain a method in which it is not necessary to remove the protein precipitant or in which it could be removed without much difficulty. Kaolin in large quantities was found appropriate for this purpose. The preliminary procedure was carried out according to Bock. The details of the whole procedure are as follows.

A measured volume of blood (30 to 50 cc.) is treated with 0.4 gm. of ground soy bean dissolved in 2 to 3 cc. of water and 1 cc. of a 3 per cent solution of NaH_2PO_4, and allowed to stand at room temperature for 30 minutes. The urea contained in the blood is converted into ammonia which is removed afterwards. One volume of this blood is slowly added to five volumes of boiling 0.01 N acetic acid in a casserole and boiled with stirring for half a minute. The same amount of boiling water is then added and the mixture is boiled with stirring for a moment, giving a clear and easily filterable precipitate. This is filtered hot through a folded filter and the casserole washed three times with 30 to 50 cc. portions of boiling water. The filtrate is rapidly concentrated over a free flame in a casserole. The residue is transferred quantitatively to a graduated cylinder or flask, making the volume nearly that of the original volume of the blood. After adding 0.1 N

acetic acid, the solution is made exactly one and a half times the volume of the blood taken. This is then shaken with kaolin (20 gm. per 100 cc.) and immediately filtered through a folded filter. The first small portion of the filtrate is usually cloudy and should be refiltered with the same filter. When in a great hurry, it is recommended to centrifuge before filtration, but in this case it is necessary to repeat the procedure again to get clear solutions. The filtrate is thus quite clear, giving no turbidity or precipitation with trichloroacetic acid or with picric acid even after standing for 24 hours, and no biuret reaction even after concentrating as far as possible. An aliquot part (30 to 60 cc.) of the filtrate is made distinctly alkaline with 1.0 N potassium hydroxide (1 to 2 cc.), the ammonia removed by boiling 2 minutes, the solution made distinctly acid with acetic acid, and concentrated to the smallest possible volume; it is then transferred to a small, accurately graduated test-tube and made to definite volume (5 to 10 cc.). The heat-kaolin method gives filtrates which rarely exhibit any tendency to froth, when shaken in the deaminizing bulb. Should frothing occur, treat again with a small portion of kaolin and measure the filtrate directly. Caprylic alcohol, as recommended for other methods, is entirely superfluous by this method.

With regard to the efficacy of the method in the recovery of added amino-acids, I first determined whether kaolin would absorb amino-acids. The following results indicate that kaolin even in a large amount does not absorb amino-acids at all (Table I).

To ascertain whether the thorough manipulation described involves losses of amino-acids, the following experiments were performed.

10 cc. of leucine solution, containing 5.42 mg. of amino nitrogen were added to 33 cc. of rabbit blood, which contained 3.81 mg. of amino nitrogen. The total amino nitrogen found was 9.07 mg., or a recovery of 5.26 mg. of amino nitrogen from the leucine. Similarly, of 13.70 mg. of amino nitrogen in 10 cc. of alanine solution, after addition to 30 cc. of blood, 13.42 mg. were recovered, and from 40 cc. of blood, 13.30 mg. were recovered. 10.18 mg. of amino nitrogen were recovered when 10.35 mg. in 10 cc. solution of amino-acids from casein were added to 30 cc. of blood.

Folin and Denis state that, "All reagents involving heating

TABLE I.*

Recovery of the Nitrogen of Amino-Acids. Amino-Acid Nitrogen per 10 Cc. of Solution.

Material.	Amount of kaolin added.	Amino-acid nitrogen.
	gm.	*mg.*
Tyrosine	0	1.67
"	1, 2, 3, or 4	1.67
Alanine	0	13.70
"	2, 3, or 4	13.70
Amino-acids from casein	0	10.35
" " "	2 or 4	10.35

* For the pure amino-acids I am indebted to Professor M. Kumagawa and his staff. Amino-acids from casein were obtained by hydrolyzing pure casein by boiling with 25 per cent hydrochloric acid for 30 hours. The liquid was neutralized with sodium hydroxide and filtered. The filtrate was treated with animal charcoal and filtered. After the solution was found entirely free from protein, it was diluted to a convenient concentration of amino-acid nitrogen and treated by the Van Slyke method. As ammonia is found in the hydrolysis of casein, it was removed before the determination of the amino-acid nitrogen.

are useless, because by heat (half an hour in a water bath) the nitrogen of normal blood filtrates may be increased to twice the real value." No figures are offered confirming this statement. Bock attempted a comparison between the direct trichloroacetic acid precipitation and the heat coagulation followed by the trichloroacetic acid precipitation, and has shown that the latter procedure gives even slightly lower results than the former. As kaolin could not be used directly for the blood protein precipitant, a comparison between the direct kaolin precipitation and the heat coagulation followed by kaolin precipitation on blood plasma was carried out. Direct kaolin precipitation was carried out as follows: The blood plasma, diluted ten to twelve times with distilled water, was acidified with 2 to 4 cc. of glacial acetic acid, 40 gm. of kaolin per 100 cc. of diluted solution were added, then the mixture was filtered with suction. After the filtrate was found entirely free from protein, an aliquot was taken and evaporated to a small volume. For the determination of amino-acids, urea and ammonia were removed by usual procedure. Tables II and III show no substantial difference between the procedures.

TABLE II.

Non-Protein Nitrogen per 100 Cc. of Blood Plasma (Determined by Kjeldahl.)

Material.	Kaolin precipitation.	Heat coagulation followed by kaolin precipitation.
	mg.	*mg.*
Beef plasma (oxalated).....	10.08	10.51
" " "	9.67	9.11
" " "	18.22	18.56
" " "	18.07	18.22

TABLE III.

Amino-Acid Nitrogen per 100 Cc. of Blood Plasma.

Material.	Kaolin precipitation.	Heat coagulation followed by kaolin precipitation.
	mg.	*mg.*
Beef plasma (oxalated).....	6.42	6.36
" " "	5.54	5.64
" " "	3.80	3.97
" " "	5.26	5.24
" " "	4.97	5.06

A comparison of the heat coagulation followed by trichloroacetic acid[5] and the heat coagulation followed by kaolin precipitation shows that there is no appreciable difference between these two methods (Table IV).

TABLE IV.

Comparison between the Heat-Trichloroacetic Acid Method and the Heat-Kaolin Precipitation. Non-Protein Nitrogen per 100 Cc. of Blood (Determined by Kjeldahl).

Material.	Heat-trichloroacetic acid.	Heat-kaolin.
	mg.	*mg.*
Beef blood (oxalated).....	14.29	14.29
Dog " "	23.78	23.54
" " "	23.88	24.18
" " "	18.21	18.21
" " "	25.50	25.50
" " "	26.90	26.45

TABLE V.

Comparison between the Heat-Trichloroacetic Acid and the Heat-Kaolin Precipitation. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Heat-trichloroacetic acid.	Heat-kaolin.
	mg.	*mg.*
Rabbit blood (oxalated)	10.49	9.82
" " "	11.68	11.68
Sheep " "	6.70	6.70
Rabbit " "	11.57	11.57
" " "	10.70	10.87
Beef " "	7.42	7.42

Alcohol precipitation has shown more or less lower values as was expected from the results of previous investigations.

TABLE VI.

Comparison between the Heat-Kaolin and Methyl Alcohol Method. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Heat-kaolin.	Methyl alcohol.
	mg.	*mg.*
Rabbit blood (oxalated)	9.72	5.89
" " "	10.55	8.68
" " "	10.67	9.53
" " "	9.17	8.99
" " "	9.81	8.49

TABLE VII.

Comparison between the Heat-Kaolin and Ethyl Alcohol Method. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Heat-kaolin.	95 per cent alcohol.
	mg.	*mg.*
Dog blood (oxalated)	8.06	5.05
Rabbit " "	7.97	5.78
" " "	7.06	5.57
" " "	11.33.	7.27
Dog " "	6.57	3.84
" " "	9.89	5.17

SUMMARY.

The use of methyl or ethyl alcohol as a precipitant for proteins in the determination of non-protein nitrogen or amino-acid nitrogen in blood is undesirable.

The methods of Greenwald and of Bock have been found serviceable for amino-acid nitrogen determination, but the procedures are somewhat troublesome.

A method is proposed (the heat-kaolin method) which has been found accurate, less troublesome, saving in time, and economical.

In conclusion I wish to acknowledge my indebtedness to Professor T. Aoyama for his generosity in allowing me to carry out this work in his laboratory and for his encouragement.

RESPIRATORY REGULATION OF THE CO_2 CAPACITY OF THE BLOOD.

I. HIGH LEVELS OF CO_2 AND ALKALI.

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The balance between acids and alkalies has come to be recognized as one of the most important of the equilibria of the living body. It is so important in fact that it appears never to be considerably altered except when the organism is practically moribund.

The two modes of estimating the degree of equilibrium which, at the present time, appear to be most interesting are by measurement of the CO_2 content of the alveolar air and of the CO_2 capacity or "alkaline reserve" of the blood (Van Slyke). The view now prevailing is that in "acidosis" the accumulation of non-volatile acids in the body neutralizes the alkalies and causes a reduction in both measurements.

In the facts and conclusions which follow we do not question the essential correctness of this general conception in conditions of true acidosis. We do however attempt to show that many conditions simulating acidosis are really due to disturbance of another factor which exerts a powerful influence upon the CO_2 capacity of the blood, and which may lead to an equilibrium at a new level. This factor is respiration. Abnormal breathing may even produce a disturbance such that equilibrium cannot be spontaneously restored. It may thus produce death.

Recent papers by Scott,[1] Peters,[2] and others have recognized certain aspects of this factor. General recognition of its power will profoundly modify the present crassly mechanistic theoreti-

[1] Scott, R. W., *Am. J. Physiol.*, 1917, xliv, 196.

[2] Peters, J. P., Jr., *Am. J. Physiol.*, 1917, xliii, 113; xliv, 84.

cal conceptions in this field, and will, we believe, find wide reaching practical applications.

In brief, the experiments to be described in this series of papers seem to indicate that when in an otherwise normal organism respiration is stimulated to excessive vigor by pain, by a drug, *e.g.*, ether (or probably by any condition which lowers the threshold and increases the sensitivity of the respiratory center), the blowing off of carbon dioxide produces a reduction in the CO_2 content of the blood. This condition very soon leads to (and in respect to C_H is probably in part compensated by) a reduction also in the CO_2 capacity (alkaline reserve) of the blood. Apparently alkali passes out of the blood into the tissues. Thus the acid-base equilibrium or CO_2: alkali balance of the blood is restored at a level much below normal, and a condition simulating, but in certain respects markedly distinct from true acidosis results. In fact, until thus compensated, the condition is a sort of low CO_2 alkalosis. It may be conveniently designated acapnia,[3] although the term thus applied has a significance much broader than heretofore. It indicates a blood low not only in CO_2 (H_2CO_3), but also in sodium bicarbonate. Unlike a true acidosis the total alkali in the body remains, so far as we can judge, unaltered; it is merely abnormally distributed.

On the other hand when the respiratory center is depressed *e.g.*, by morphine, or when by the administration of high concentrations of CO_2 in the air breathed, the alveolar CO_2 is raised, the CO_2 content of the blood is also raised. This causes (and in respect to the C_H of the blood is probably compensated by) an increase in the CO_2 capacity or "alkaline reserve" of the blood, due apparently to the passage of alkali from the tissues into the blood. Thus a pseudoalkalosis or, if one thinks only in terms of C_H, a CO_2 acidosis, is produced. It may conveniently be termed hypercapnia: both the CO_2 and the sodium bicarbonate in the blood are above normal. Probably the total alkali in the body is unaltered. It is merely redistributed.

Either of these conditions, when once induced, shows a distinct tendency to be maintained. Any rapid variation from the new

[3] Henderson, Y., *Am. J. Physiol.*, 1908, xxi, 126; 1908–09, xxiii, 345; 1909, xxiv, 66; 1909–10, xxv, 310, 385. Henderson, Y., and Scarbrough, M. McR., *ibid.*, 1910, xxvi, 260. Henderson, *ibid.*, 1910–11, xxvii, 152.

level of CO_2 and alkali back toward the normal level is resisted by the action of the respiratory center or allowed to develop only gradually.

In this paper we shall give the experiments in which a high level of CO_2 and alkali, or hypercapnia, was induced. In succeeding papers experiments producing low levels of CO_2, or acapnia, will be presented.

Theoretical.

Attention at present is fixed so exclusively upon C_H that we must specifically point out that actually this "physiological constant" only varies imperceptibly. Even these slight variations induce powerful counteracting efforts. Through these efforts nearly normal equilibrium in respect to C_H is attained at highly abnormal levels of CO_2: alkali. Thus, as we see it, interest should now shift, at least for the physiologist, from C_H and the physicochemical equilibrium to these levels of CO_2:alkali and the physiological equilibrium.

The physicochemical equilibrium (L. J. Henderson) is approximately the balance of CO_2 against $NaHCO_3$ to maintain $\overset{+}{H}$, that is, C_H, in the expression:

Pulmonary air.	Blood.
CO_2	$\rightleftharpoons$ Dissolved$CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons \overset{+}{H} + \overset{-}{HCO_3}$
	$\overset{+}{Na} + \overset{-}{HCO_3} \rightleftharpoons NaHCO_3$

On the other hand the physiological equilibrium and the respiratory activity by which any great and sudden changes in C_H are prevented may be expressed, for example, at three levels of CO_2 and alkali as shown in the following table.

The numerator (H_2CO_3) is always proportional to the alveolar CO_2 because the amount of CO_2 dissolved (not combined with alkali) is determined by the tension of CO_2 in the alveolar air (law of Henry). The alveolar CO_2 is of course always inversely proportional to the pulmonary ventilation per unit mass of CO_2 eliminated.

Blood Plasma.		C_H	Respiration.		Condition.
CO_2 in form of			CO_2 in alveolar air.	Pulmonary ventilation in cc. of air per 5.5 cc. CO_2 eliminated.	
$H_2CO_3+NaHCO_3$	$\frac{H_2CO_3}{NaHCO_3}$				
vol. per cent	*vol. per cent*		*per cent*	*cc.*	
63	3:60	Normal.	5.4	100	Normal level of CO_2: alkali.
42	2:40	"	3.6	150	Low level of CO_2: alkali.
84	4:80	"	7.2	75	High level of CO_2: alkali.

The expression "level of CO_2 and alkali" does not mean the proportion of CO_2 to alkali but the quantity of both; that is, their sum. Thus when in the plasma the proportion of H_2CO_3:$NaHCO_3$ is 3:60 the level of the equilibrium (C_H) is normal or nearly normal at 63. When it is 2:40 the equilibrium is at the low level of 42, while 4:80 is at the high level of 84. In all these cases the proportion of H_2CO_3:$NaHCO_3$ is equal to 1:20 and the C_H may (theoretically) be the same; that is, normal (Van Slyke and Cullen[4]). The breathing to maintain this equilibrium must, for a definite CO_2 elimination, be 50 per cent greater than normal at the low level and 25 per cent less than normal at the high level. Whenever the proportion is forced away from 1:20 (or its equivalents in respect to C_H 3:60, 4:80, 2:40) the system seeks by physiological means to reattain equilibrium, in as nearly the normal C_H as possible, at a new level. If the proportion is more than 1:20 the C_H is above normal and if less than 1:20 it is below normal (cf. Peters).[2]

These figures apply to plasma, in which, at least for theoretical discussion, the conditions are comparatively simple. For whole blood these figures should be somewhat smaller (about five-sixths). For the purposes of our experiments analyses of plasma would however be much less significant than whole blood.

In the production and maintenance of the low level as it exists probably in acidosis of renal or metabolic origin the alkali of the

[4] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 294.

blood by influencing the respiratory center controls the CO_2 (H_2CO_3). On the other hand in the low and high levels produced by disturbances of breathing the respiratory center by controlling the CO_2 exerts a powerful influence upon the alkali ($NaHCO_3$).

If objection be taken to our saying that C_H may be "normal" at various levels of CO_2, we would point out that people living 5,000 or 10,000, or even 15,000 feet above sea level, or again below sea level, have as much right to consider themselves normal as have we who live by the sea. But their levels of CO_2 are widely different from ours.[5]

Method.

All experiments were performed on healthy dogs. The femoral artery was exposed under local anesthesia (cocaine 0.5 cc. of 2 per cent solution) and a cannula inserted. Samples of blood, 3 to 6 cc. each, were withdrawn at regular intervals with proper precautions to avoid getting stagnant blood. Clotting was prevented by a small amount of powdered potassium oxalate. 1 cc. of the whole blood was analyzed to determine its CO_2 content. The remainder of the sample was then brought into equilibrium at 20°C. with air containing 5.4 to 5.7 per cent CO_2 (determined by analysis). 1 cc. of this whole blood was then analyzed. This gave the CO_2 capacity. The methods were those described by Henderson and Morriss.[6] The figures for CO_2 capacity are proportional and closely approximating but not absolutely identical with those which would be computed by the Van Slyke method. We prefer the expression "CO_2 capacity," to the still rather hypothetical expression "alkaline reserve." The capacity of the proteins to combine with CO_2 may be considerable (Buckmaster[7]). By CO_2 capacity we mean the total amount of CO_2 (reduced to 0° and 760 mm.) in the blood whether in solution or combined with alkali or protein at the tension of the CO_2 in the air with which the sample of blood is brought into equilibrium.

The alveolar air was determined with a mask and bag by the Higgins-Plesch method.

[5] Fitzgerald, M. P., *Phil. Tr. Roy. Soc., Series B*, 1913, cciii, 351; *Proc. Roy. Soc., Series B*, 1914–15, lxxxviii, 248.

[6] Henderson and Morriss, *J. Biol. Chem.*, 1917, xxxi, 217.

[7] Buckmaster, G. A., *J. Physiol.*, 1917, li, 164.

The reliability of all our work depends upon the accuracy with which the blood was "saturated" or (better) "equilibrated" with air containing 5.5 per cent CO_2. The method of equilibration and analysis was therefore subjected to the following tests.

A supply of arterial blood was drawn. Analysis was made for (*a*) the CO_2 content. A sample was then equilibrated with air containing 5.5 per cent CO_2 and analyzed (*b*). A second portion of the blood was then equilibrated with air containing 10 per cent CO_2. Part was analyzed (*c*), and the rest was then equilibrated with 5.5 per cent CO_2 and analyzed (*d*). A third sample was equilibrated with atmospheric air. A part of this was then analyzed (*e*), and the remainder was equilibrated with 5.5 per cent CO_2 and analyzed (*f*). In all cases the equilibration was carried out by the method used in routine determinations of the CO_2 capacity. The equilibrating air was passed through a tube into a test-tube containing 2 or 3 cc. of blood under albolene until the liquid was violently agitated for 30 seconds and for the most part in foam. The test-tube was then stoppered and set aside until the blood and oil separated.

The foregoing series of equilibrations and analyses was repeated with blood drawn from the same dog several hours later. The equilibrations lasted 2 minutes each. The results obtained were as follows:

	a	*b*	*c*	*d*	*e*	*f*
First series.....	47	50	60	51	37	50
Second "	47	47	58	47	37	49

Under the extreme conditions the concordances 50, 51, 50 in one series and 47, 47, 49 in the other (*b*, *d*, and *f*) are satisfactory.

Object of the Experiments.

Our aim was to determine the effect upon the CO_2 capacity of the blood induced by increasing the CO_2 tension of the pulmonary air. This was effected by depressing respiration by means of morphine or by administering CO_2 in proper dilution in the air breathed. For the latter purpose a Douglas bag of 50 liters capacity was filled with air to which 5 or 6 per cent of CO_2 was added. The animal rebreathed this mixture through a

closely fitting mask and as the CO_2 increased oxygen was added in equal amount.

EXPERIMENTAL.

Two control experiments were performed. They demonstrate that under the conditions all the functions remain nearly constant. Even in the slight variations observed the CO_2 content of the blood shows a tendency to follow the alveolar CO_2 within 5 minutes. With a distinct lag of 15 to 30 minutes the CO_2 capacity follows the CO_2 content.

Experiment 1.—Control. Dog, male, 12 kilos. No drug. Animal quiet and comfortable, and breathing normally throughout. Air used for equilibrating blood contained 5.4 per cent CO_2.

Time.	Conditions.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
p.m.		*per cent*	*vol. per cent*	*vol. per cent*	°C.
4.15	Normal.	5.4	46	47	39.8
4.45	"	5.5	48	47	39.8
5.15	"	5.2	44	45	39.8
5.45	"	5.4	50	47	39.7
6.15	"	5.2	50	50	39.8
6.45	"	5.3	48	46	39.8
7.15	"	5.4	49	50	39.6

Experiment 2.—Control. Dog, male, 9 kilos. Conditions as in Experiment 1. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	°C.
10.15 a.m.	Normal.	5.3	47	49	39.7
10.45 "	"	5.2	47	48	39.7
11.15 "	"	5.4	50	48	
11.45 "	"	5.1	48	48	39.7
12.15 "	"	5.5	51	50	
12.45 "	"	5.5	51	49	39.8
1.45 p.m.	"	5.4	51	52	
2.15 "	"	5.4	50	50	39.7

In the next four experiments, after a preliminary normal period in each case, morphine was administered subcutaneously. The extent of the depression of respiration may be judged by the rise in the alveolar CO_2 from the normal of about 5.3 up to amounts ranging from 6.1 to 6.9. In one case (Experiment 6) this rise was comparatively slight at first (owing to the animal being of an excitable character and breathing correspondingly) and in the columns showing the CO_2 content and CO_2 capacity the effects are correspondingly slight. Later in this experiment and in all of the experiments in which the alveolar CO_2 attained a high level, the CO_2 content of the blood invariably followed it, and the CO_2 capacity followed the CO_2 content with a lag of 20 to 30 minutes. Thus the CO_2 content rose from about 50 up to values ranging from 63 to 67, and the CO_2 capacity from about 50 up to values of 59 to 64.

Experiment 3.—Dog, male, 14 kilos. Morphine administration, depressed respiration, followed by rise of CO_2 content and CO_2 capacity. Equilibrating air 5.7 per cent CO_2.

Time.	Condition.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	°C.
9.30 a.m.	Normal.	5.3	52	52	39.9
10.00 "	"		52	53	
10.05 "	Morphine, 0.14 gm.				
10.15 "	Vomiting and panting.				
10.40 "	Quiet.	6.6	57	48	
11.20 "			60	53	37.8
12.00 "	Quiet.	6.6	62	54	
12.30 "	"		62	56	
1.30 p.m.	"	6.9	67	59	
2.10 "	"		63	60	36.6
2.40 "	"		58	59	
3.30 "	"	6.8	64	62	
6.00 "	Struggling.	4.8	49	56	37.0

Experiment 4.—Dog, male, 9 kilos. Morphine administration. Conditions as in Experiment 3. Equilibrating air 5.7 per cent CO_2.

Time.	Condition.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	*°C.*
11.30 a.m.	Normal.	5.4	49	50	39.7
11.35 "	Morphine, 0.16 gm.				
12.15 "		5.9	57	54	
12.40 "		6.0	60	56	38.8
1.20 p.m.		6.3	62	59	
2.00 "		6.7	64	59	
2.40 "		6.6	63	60	38.6

Experiment 5.—Dog, male, 12 kilos. Morphine administration. Conditions as in Experiment 3. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	*°C.*
10.15 a.m.	Normal.	5.3	50	51	39.6
10.20 "	Morphine, 0.24 gm.				
10.50 "		6.0	56	54	
11.35 "		6.6	64	58	38.4
12.30 "			63	60	
1.30 p.m.		6.7	65	59	
3.00 "	Very quiet.		70	64	

Experiment 6.—Dog, male, 10 kilos. Morphine administration. Conditions as in Experiment 3. Equilibrating air 5.4 per cent CO_2.

Time.	Condition.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	*°C.*
10.00 a.m.	Normal.	5.4	49	49	39.8
10.30 "	Morphine, 0.16 gm.				
11.15 "			54	48	
12.00 "		5.8	56	52	39.0
1.15 p.m.			60	56	
2.00 "		6.1	63	58	
3.00 "			64	58	
4.00 "			62	59	38.8

In Experiment 7 morphine was administered and the animal was then made to rebreathe (by means of a mask and Douglas bag) air of a CO_2 content at first practically the same as the normal alveolar air and rising gradually to 8.2 per cent. The CO_2 content of the blood rose in 1 hour and 10 minutes to 76 and the CO_2 capacity to 67.

Experiment 7.—Dog, male, 8 kilos. Morphine and CO_2 administration. Procedure same as Experiments 3 to 6 except that dog rebreathed into a large bag, which in the beginning contained 5 per cent CO_2. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Bag of air CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
p.m.		*per cent*	*vol. per cent*	*vol. per cent*	*°C.*
3.35	Normal.		48	50	39.9
4.00	Morphine, 0.12 gm.				
4.20	Breathing CO_2.	5.1			
4.40	" "		70	56	
5.10	" "		69	64	40.0
5.15	" "	8.2			
5.30	" "		76	67	
6.00	" "		74	66	

Finally three experiments were performed in which no morphine was administered, but the rebreathing from the bag was begun with about 6 per cent of CO_2 and continued until the CO_2 had accumulated up to 10, 11, and 20 per cent respectively. (Ample oxygen was supplied.) Of course the breathing was stimulated to violent activity at percentages between 6 and 10, but much less above 10 per cent. The effects on the blood were of exactly the same character as in the experiments in which respiration was depressed and the alveolar CO_2 raised by means of morphine. The CO_2 content was forced up (to 74, 73, and 87) and the CO_2 capacity followed it upward (to 64, 59, and 74). When the rebreathing was stopped and the animal again allowed to breathe normal air, the CO_2 content led the CO_2 capacity downward toward normal values again, but the descent was slow and gradual.

Experiment 8.—Dog, male, 8 kilos. CO_2 administration. Procedure same as Experiment 7, except that morphine was omitted. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Bag of air CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
p.m.		*per cent*	*vol. per cent*	*vol. per cent*	*°C.*
4.15	Normal.		51	50	39.8
4.30	CO_2 started.	6.0			
4.45			54	55	
5.15		10.2	68	59	39.9
5.45		11.4	72	64	
6.15			74	63	
6.20	CO_2 stopped.				
6.45			64	67	

Experiment 9.—Dog, female, 14 kilos. CO_2 administration. Procedure similar to Experiment 8. Equilibrating air 5.4 per cent CO_2.

Time.	Condition.	Bag of air CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
p.m.		*per cent*	*vol. per cent*	*vol. per cent*	*°C.*
1.40	Normal.		48	46	39.8
2.15	CO_2 started.	6.0			
2.45		8.2	62	54	39.7
3.15			66	56	39.6
3.45		10.3	73	59	
4.15			72	61	39.8
4.20	CO_2 stopped.				
4.45			54	59	
5.15			50	54	
5.45			47	52	

Experiment 10.—Dog, male, 11 kilos. CO_2 administration. Procedure same as Experiments 8 and 9, but the CO_2 in bag was run up higher. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Bag of air CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	*°C.*
11.30 a.m.	Normal.		48	50	39.9
11.45 "	CO_2 started.	6.2			
12.15 "			57	54	
1.15 p.m.			67	60	
2.15 "		10.3	70	62	40.1
2.45 "			72	66	
3.15 "		14.0	79	65	
4.00 "			87	71	
4.45 "		20.0	86	74	42.0
4.50 "	CO_2 stopped.*				

* After drawing a few breaths of fresh air the dog stopped breathing. The heart continued to beat for several minutes and then stopped. Died.

Experiment 10 is extraordinary. The CO_2 in the air breathed reached 20 per cent. The CO_2 content of the blood rose to 86 and the CO_2 capacity to 74. When the animal was finally allowed to breathe normal air again its respiration ceased after a few breaths and a fatal apnea followed. Apparently the alkali content of the blood had been raised so high that when the abnormally high pressure of CO_2 was withdrawn the respiratory center lacked an adequate stimulus.

CONCLUSIONS.

Whenever by means of morphine respiration is depressed and the alveolar CO_2 is raised, or whenever by the administration of CO_2 in the air breathed the CO_2 content of the blood is forced up, the CO_2 capacity (alkaline reserve) of the blood also rises. This rise is probably due to a compensatory passage of alkali from the tissues into the blood. Through this mechanism respiration exerts a powerful influence upon the "alkaline reserve" of the blood.

RESPIRATORY REGULATION OF THE CO_2 CAPACITY OF THE BLOOD.

II. LOW LEVELS OF CO_2 AND ALKALI INDUCED BY ETHER. THEIR PREVENTION AND REVERSAL.

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In the light of present knowledge it appears probable that many, perhaps most, of the functional disturbances induced by anesthesia are due to the production of an abnormal CO_2 capacity (alkaline reserve) in the blood, or to deeper effects of which the CO_2 capacity is an index.

At present such effects are generally interpreted as "acidosis." Our observations show that, at least under ether, they are of a very different origin.

Morriss[1] has shown that in patients under ether a lowering of the alkaline reserve of the plasma occurs. On the basis of some preliminary experiments on dogs Prince and the authors[2] have verified Morriss' observation and have obtained results indicating that the effect is due to the respiratory excitement induced by ether. An abnormally great amount of CO_2 is ventilated out of the blood by the excessive breathing and the CO_2 content is thus lowered. Sometimes when this blowing off has been very rapid the result is failure of respiration: apnea vera. When the blowing off is less rapid but is prolonged another change occurs which (as Prince first pointed out to us) is of a compensatory character. This consists (as set forth in general terms in the first paper of this series) in a decrease of the CO_2-combining power (alkaline reserve) of the blood. The simplest supposition upon which to explain it is that when the alkalinity

[1] Morriss, W. H., *J. Am. Med. Assn.*, 1917, lxviii, 1391.

[2] Henderson, Y., Prince, A. L., and Haggard, H. W., *J. Am. Med. Assn.*, 1917, lxix, 965.

of the blood is abnormally increased by the blowing off of CO_2, alkali passes out of the blood into the tissues. As a result of these two processes both the CO_2 and the alkali in the blood are decreased, and the proportion of H_2CO_3:$NaHCO_3$ and consequently the C_H of the blood are much less decreased than would be the case if only the CO_2 were altered.

If the "level of CO_2 and alkali" (as defined in the previous paper) is reduced below the critical value lying between 33 and 36 volumes per cent of CO_2, a condition of general depression of all vital functions results. Above the critical level the process appears to be reversible, for if CO_2 is administered in the air breathed and the CO_2 content of the blood is thus raised the CO_2 capacity follows it upward, probably because of the passage of the alkali from the tissues back into the blood. On the other hand if the marked resistance to further depletion of CO_2 capacity which occurs at the critical level is broken down and the CO_2 capacity further reduced, the result is a condition of vital depression from which the subject does not spontaneously recover. This condition may be termed acapnial shock.

In the experiments to be reported here the methods were identical with those in the first paper of this series, and the controls reported there apply equally here.

EXPERIMENTAL.

In the first experiment are shown the effects (in the dog) of careful and skilful etherization by the open method. The initiation of anesthesia was rapid, the administration as nearly uniform as practicable by this method, and both excitement of respiration from too little ether and depression from too much were avoided. The CO_2 content was reduced in the course of the 1st hour from the normal of 50 down to 45. It underwent no considerable change during the succeeding 5 hours.

Experiment 1.—Dog, male, 11 kilos. Skilful etherization. Full anesthesia by open method. Equilibrating air 5.6 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
			vol. per cent	*vol. per cent*
8.30 a.m.	Normal.	21	51	50
9.00 "	Ether.			
9.30 "		47	43	50
10.00 "		48	43	45
10.30 "		26	46	46
11.00 "		30	51	48
11.30 "		17	47	47
12.00 "		19	44	47
12.30 "	Shallow respirations.	29	56	48
1.00 p.m.	Less ether.	21	46	45
1.30 "		14	48	44
2.00 "		19	50	44
2.30 "		29	44	46
3.00 "		25	41	45
3.05 "	Experiment stopped.	Dog in good condition.		

In the second experiment the initiation of anesthesia was purposely made unskilfully slow. The dog was allowed to struggle in the stage of excitement for a considerable time, and when this period came to an end the ether was given irregularly so that at no time was there deep anesthesia.

Experiment 2.—Dog, female, 8 kilos. Etherization, open method, excessive respiration. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
p.m.			*vol. per cent*	*vol. per cent*
4.00		18	47	49
4.24	Ether started.			
4.26		44		
4.28		68		
4.30		74		
4.33		90		
4.35			38	48
5.00		62		
5.10			39	44
5.30		34		
6.10			40	43
6.30		46		
7.10			38	41

In the third experiment is afforded an example of extremely bad light etherization, yet which was not so bad as to cause early death from failure of breathing (apnea). The excessive breathing reduced the CO_2 content of the blood progressively and the CO_2 capacity followed downward with the usual lag until at a CO_2 capacity of 28 the animal was moribund. It died soon after.

Experiment 3.—Dog, male, 14 kilos. Prolonged ether excitement. Equilibrating air 5.4 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
			vol. per cent	*vol. per cent*
9.20 a.m.	Normal.	19	54	51
9.30 "	Ether started.			
10.00 "		63	44	52
10.30 "		82	39	44
11.00 "		70	36	43
11.30 "		78	37	44
12.00 "		42	36	41
12.30 "		84	35	39
1.00 p.m.		59	37	38
1.30 "		76	31	36
2.00 "		56	22	37
2.30 "		59	24	31
3.00 "		82	27	28
3.15 "	Moribund. Ether stopped.			
3.19 "	Stopped breathing.			
3.24 "	Heart stopped. Dog dead.			

In the fourth experiment the same procedure was followed until the CO_2 content had fallen at the end of 210 minutes to 35 volumes per cent and the CO_2 capacity to 38. The administration of ether was then stopped, and in the course of the succeeding 90 minutes the CO_2 content rose again nearly to normal and thereby caused the CO_2 capacity to rise to 46, only a little below the normal level.

Experiment 4.—Dog, male, 10 kilos. Light and irregular ether for 210 minutes, with spontaneous recovery toward normal during following 90 minutes. Equilibrating air 5.7 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
			vol. per cent	*vol. per cent*
9.30 a.m.		20	50	53
9.45 "	Ether started.			
10.00 "		82		
10.15 "			42	50
10.30 "		68		
10.45 "			36	47
11.00 "		66		
11.15 "			37	44
11.30 "		69		
11.45 "			36	41
12.00 "		59		
12.15 "			40	38
12.30 "		60		
1.15 p.m.			35	38
1.20 "	Ether stopped.			
1.45 "			43	39
2.15 "			49	43
2.45 "			47	46
2.50 "	Animal in good condition.			

Evidently in the foregoing experiment the decrease of the CO_2 capacity (alkaline reserve) was spontaneously reversible from the low level reached.

The first part of Experiment 5 was similar to the preceding experiment. After the CO_2 capacity had been reduced to 39 in this case however, instead of discontinuing the ether the amount administered was increased to a maximum just short of causing respiratory failure. This condition was continued for 80 minutes. Under the depression of respiration thus caused the alveolar CO_2 was of course increased. The CO_2 content of the blood rose to 67 and the CO_2 capacity was raised to 54; that is, slightly above normal. The animal died soon after, probably from an insufficiency of oxygen.

Experiment 5.—Dog, male, 12 kilos. Light and irregular etherization followed by very deep etherization (with no rebreathing). Equilibrating air 5.65 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
p. m.			*vol. per cent*	*vol. per cent*
3.00		21	49	50
3.15	Ether started.			
3.45		82	39	47
4.15		73	37	42
4.45		57	38	43
5.15		61	36	40
5.45		63	39	39
6.00	Ether increased to limit.			
6.15		47	43	39
6.45	Blood dark.	37. shallow.	53	41
7.15	" "	46 "	67	54
7.23	Dog dead.			

From this experiment it is clear that when the CO_2 content is lowered by etherization the effect is not due to any influence of the ether *per se* other than the excessive pulmonary ventilation and low alveolar CO_2. In order however to make absolutely sure of this point we performed Experiments 6 and 7. We reasoned that if ether acts in some obscure way upon metabolism to induce "acidosis" (the opinion now widely held), then the more ether administered the greater should be the so called acidosis and the lower the CO_2-combining power. As ether in low concentrations excites and in high concentrations depresses respiration, it was easy to frame a crucial experiment. Thus in Experiments 6 and 7 an etherization so profound was maintained that respiration was depressed and the CO_2 content rose instead of falling. As the protocols show the CO_2 capacity followed upward, rising from 50 to 61 in one case, and from 47 to 60 in the other.

Experiment 6.—Dog, male, 9 kilos. Profound etherization and depressed respiration. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
p. m.			*vol. per cent*	*vol. per cent*
3.30	Normal.	16	52	50
3.45	Ether started, pushed rapidly.			
4.15		11	60	54
4.30	Shallow respirations. Blood dark.	16	57	56
4.45	" " " "	22	57	55
5.15	" " " "	8	60	61
5.20	Breathing stopped. Dog dead.			

Experiment 7.—Dog, male, 7 kilos. Similar to Experiment 6. Equilibrating air 5.6 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
p. m.			*vol. per cent*	*vol. per cent*
6.10	Normal.	21	47	47
6.30	Ether started. Administered rapidly.			
7.00	Respirations very shallow.	72, blood dark.	64	63
7.05	Ether lightened.			
7.30	Shallow respirations.	32 " "	59	60
7.45	" "	40 " "	63	60
7.50	Dog in critical condition. Ether stopped.			
7.52	Three or four more breaths, then breathing stopped, heart beating.			
7.56	Heart stopped. Dog dead.			

Even Experiments 6 and 7, decisive as they appear in contrast to those preceding them, *e.g.*, Nos. 2, 3, 4, and 5, might leave open the possibility that the excitement of light ether causes a depression of the CO_2 capacity in some way (*e.g.*, excessive adrenal secretion) other than by the blowing off of CO_2. Accordingly we performed Experiments 8 and 9 in which light etherization was maintained for several hours but loss of CO_2 was prevented by the fact that the air which the animal breathed was enriched by the addition of CO_2 in the amounts shown in the protocols. It will be seen that in both cases the CO_2 capacity

was slightly elevated and that when the experiments were discontinued, after $5\frac{1}{2}$ and $4\frac{1}{4}$ hours respectively, the animals were in excellent condition.

Experiment 8.—Dog, male, 9 kilos. Light etherization with CO_2 inhalation. Tracheal cannula connected with closed circuit ether bottle. Air containing approximately 7 per cent of CO_2 administered through ether. Dog at all times was kept under very light ether as determined by periods of struggling, vigorous breathing, and full reflexes. Equilibrating air 5.45 per cent CO_2.

Time.	Condition.	CO_2 breathed.	Blood CO_2.		Arterial pressure.
			Content.	Capacity.	
p. m.		*per cent*	*vol. per cent*	*vol. per cent*	*mm.*
3.30	Normal.	0	49	51	
4.00	Under ether.	0	42	49	170
4.05	CO_2 started.	7.5			
4.15		7.5	58	50	168
4.45		7.1			
5.00		3.8	52	55	150
5.30		7.7	63	57	150
6.15		7.6	55	55	154
6.45		7.0	60	57	160
7.15		6.8	56	56	150
7.45		6.8	53	54	160
	Dog in excellent condition.				

Experiment 9.—Dog, male, 14 kilos, young. Similar to Experiment 8. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	CO_2 breathed.	Blood CO_2.		Arterial pressure.
			Content.	Capacity.	
p. m.		*per cent*	*vol. per cent*	*vol. per cent*	*mm.*
2.48	Normal.	0	48	47	
3.15	Ether started.				
3.42		0			128
3.45	CO_2 started.	6.2			
4.45		6.2	63	56	116
5.45		6.0	68	54	110
6.45		7.7	71	51	114
7.45		6.4	62	56	110
8.45		7.1	67	55	108
	Dog in good condition. Blood a good bright normal color.				

CONCLUSIONS.

The disturbance of the CO_2 capacity of the blood by ether appears from these experiments to be wholly dependent on disturbance of respiration. If the anesthesia is managed so that respiration is but little increased, the lowering of the CO_2 capacity of the blood is slight. Ether hyperpnea however causes a very great reduction. Down to the critical level between 33 and 36 volumes per cent the process is spontaneously reversible and the animal recovers. Below this level it appears to be irreversible, and death ensues.

At present such effects even above the critical level are generally interpreted as "acidosis." Our results show them to be of a very different origin. It is possible however that below the critical level a true acidosis of an asphyxial character sets in.

Etherization so profound as to depress respiration causes a rise of the CO_2 capacity.

Light etherization such as is otherwise most effective in lowering the CO_2 capacity, and most harmful to the subject, loses this influence when administered with sufficient CO_2 to maintain the alveolar CO_2 at a normal level.

From these facts it is clear that under ether the CO_2 capacity of the blood (alkaline reserve) follows and is controlled by the CO_2 content, and that the CO_2 content is in turn dependent upon the alveolar CO_2, which is determined by the breathing.

These results afford, we believe, final proof of the essential correctness of the views on this topic (acapnia under anesthesia[3]) which have been advocated now for 10 years past in papers from this laboratory.

We desire to acknowledge our debt to Van Slyke and his collaborators[4] for bringing forward the conception of the "alkaline reserve," or as we prefer to call it the CO_2 capacity of the blood. This conception has enabled us to produce proof on a topic which after controversy and general rejection was evidently passing into oblivion.

[3] Bryant, J., and Henderson, Y., *J. Am. Med. Assn.*, 1915, lxv, 1 (discussion of clinical aspects of problem and references to previous work).

[4] Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 401.

RESPIRATORY REGULATION OF THE CO_2 CAPACITY OF THE BLOOD.

III. THE EFFECTS OF EXCESSIVE PULMONARY VENTILATION.

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Pain causes excessive breathing: abnormally intense pulmonary ventilation. What part does this ventilation play as an intermediary between pain and its end results; *e.g.*, the characteristic alterations of the CO_2 capacity of the blood, of venous and arterial pressure, of oxygen consumption, etc.? For an answer to this question we need experiments which afford excessive pulmonary ventilation but none of the other conditions of pain. In man this test may be made experimentally (so far as one dares and is physically able) by prolonged voluntary forced breathing.[1] In animals the problem may be attacked by means of excessive artificial respiration. In this paper we shall report some experiments on the latter line of attack.

There seems to be a misapprehension as to what we mean by excessive breathing. One observer writes us from a casualty clearing station in France as follows:

"As to excessive breathing, we have made careful observations on freshly wounded men both in the trenches and in the casualty clearing station, and have found that *it does not occur*. A man was brought in a few minutes after he had been hit by a small splinter of a bomb which had penetrated his wrist. He was suffering intense pain and was breathing, each expiration a grunt, twelve times a minute."

We have tried breathing in this way ourselves and find that after doing so for 40 seconds an apnea of 20 seconds followed. Quantitatively this indicates that, at least as we did it, such breathing involves a 50 per cent overventilation. The same writer says, "The severely wounded commonly testify to feeling no pain." This, we think, corresponds with the

[1] Henderson, Y., *Am. J. Physiol.*, 1909–10, xxv, 321; *Bull. Johns Hopkins Hosp.*, 1910, xxi, 236; *J. Pharm. and Exp. Therap.*, in press.

fact that, as reported, shock occurs in only a minority of the wounded. Furthermore overventilation quickly results in a numbing of sensation,—an almost anesthetic condition as regards consciousness, and yet the excessive breathing may continue.

Method.

In general the methods employed were similar to those in the preceding papers of this series. The animals were however given an intramuscular injection of chloratone dissolved in alcohol. At the outset of each experiment they were also etherized for a few minutes.

The apparatus with which the excessive artificial respiration was administered is shown in Fig. 1. It consisted of two rotary air blowers belted to a shafting (not shown) so that one forced air toward a Y tube tied into the trachea and the other sucked air from it. Escape valves were connected with each side to prevent the pressures either positive or negative ever exceeding 30 cm. water column. By means of a hand lever the Y tube in the trachea was opened alternately to pressure and suction.

EXPERIMENTAL.

In a control experiment without artificial respiration it was found that chloratone itself in the dosage employed has no marked effect upon either the CO_2 content or CO_2 capacity of the blood. It is well known that dogs under chloratone have a low arterial pressure.

Experiment 1.—Control. Dog, male, 19 kilos. Chloratone anesthesia. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Blood CO_2.	
		Content.	Capacity.
		vol. per cent	*vol. per cent*
10.30 a.m.	Normal.	48	49
10.45 "	Chloratone administered, 4.8 gm.		
11.35 "		46	48
1.35 p.m.		47	46
2.35 "		48	46

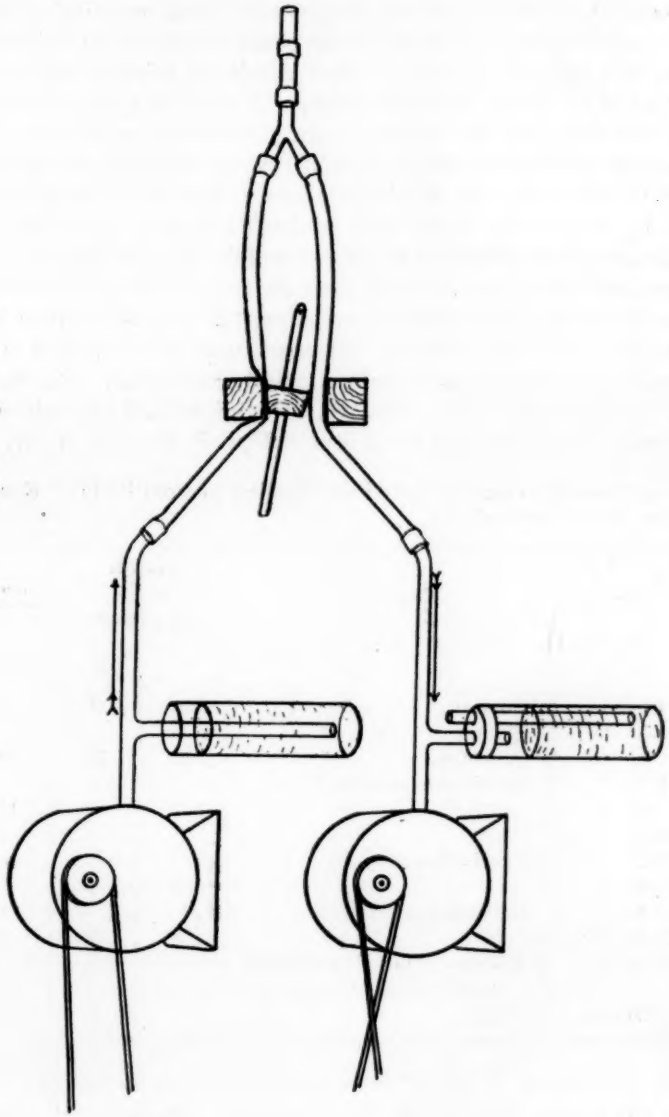

Fig. 1.

In Experiment 2 the artificial respiration was made as vigorous as possible. The instant the lungs were filled or emptied the lever was reversed and the alternate phase of respiration induced. From the protocol it appears that within 22 minutes the CO_2 content of the blood was reduced from 52 to 22, the CO_2 capacity from 50 to 38, and the arterial pressure from 130 mm. down to 50. When the ventilation was stopped it was necessary to administer artificial respiration by hand by squeezing the chest occasionally in order to prevent death from prolonged apnea. Even so, all of the functions measured continued to fall, the CO_2 capacity to 28 and arterial pressure to 25, and the animal died 55 minutes after the artificial respiration was stopped. In all of these experiments a careful autopsy was conducted, air embolism and pulmonary hemorrhages being particularly looked for. The findings in this respect were negative. In this animal the only abnormality found was a mass of roundworms in the right heart.

Experiment 2.—Dog, male, 8 kilos. Passive overventilation. Equilibrating air 5.6 per cent CO_2.

Time.	Condition.	Blood CO_2.		Arterial pressure.
		Content.	Capacity.	
		vol. per cent	*vol. per cent*	*mm.*
10.15 a.m.	Normal.	53	51	
10.30 "	Chloratone, 2 gm.			
11.35 "	Operated.	52	50	130
11.43 "	Ventilation started.			
11.50 "				110
11.55 "				50
12.05 "	Ventilation stopped.			
12.06 "		22	38	
12.20 "	Reflexes hyperactive.			25
12.30 "		20	28	25
12.35 "	Reflexes practically absent. Respirations 36 per min.			
1.00 p.m.	Dead.			

In Experiment 3 the artificial respiration was carried on much less vigorously than in the preceding case, but was still markedly in excess of normal breathing. In the course of an hour and

45 minutes the CO_2 content of the blood was reduced to 28 volumes per cent, the CO_2 capacity to 34, and the arterial pressure to 70 mm. After termination of the mechanical artificial respiration it was found necessary for a time to squeeze the chest occasionally to prevent death in apnea.

In the course of half an hour spontaneous respiration of a shallow rapid type returned, but the animal continued to sink and died an hour and 40 minutes after the cessation of the period of excessive ventilation.

In this and in the preceding experiment it was observed that at first the reflexes, particularly the knee-jerk, seemed to be actually increased in responsiveness, but that after the excessive artificial respiration was stopped the reflexes decreased in responsiveness until they had practically disappeared.

Experiment 3.—Dog, male, 7 kilos. Passive overventilation, similar to Experiment 2, but not so active. Equilibrating air 5.6 per cent CO_2.

Time.	Condition.	Blood CO_2.		Arterial pressure.
		Content.	Capacity.	
p. m.		*vol. per cent*	*vol. per cent*	*mm.*
3.00	Normal.	55	56	
3.20	Chloratone, 1.4 gm.			
4.50		54	55	130
5.15	Ventilation started.			
5.30		37	41	130
6.00		31	37	120
6.15	Reflexes hyperactive.			
6.30		31	35	95
7.00		28	34	70
7.05	Ventilation stopped. Artificial respirations 3 min. (manual).			
7.30	Reflexes partially abolished.			
7.30		23	38	50
8.00	Respirations shallow and rapid, 44 per minute.			
8.00				50
8.30	Respirations, 55 per min.			50
8.35		26	35	48
8.47	Dog dead.			

Autopsy.—No pulmonary lesions. No air emboli in coronary vessels.

Experiment 4 affords evidence that excessive ventilation may have profound effects, including circulatory failure, which may continue even when the CO_2 capacity returns to normal,—unless perhaps this animal was unusually susceptible to chloratone. Death was evidently due to circulatory failure.

Experiment 4.—Dog, male, 9 kilos. Overventilation. Similar to Experiment 3. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Blood CO_2.		Arterial pressure.
		Content.	Capacity.	
		vol. per cent	*vol. per cent*	*mm.*
9.00 a.m.	Normal.	47	50	
9.20 "	Chloratone, 1.8 gm.			
10.20 "		49	48	100
10.35 "	Ventilation started.			
10.50 "		29	42	70
11.20 "		21	37	70
11.50 "		34	40	60
12.20 "	Reflexes hyperactive.			
12.20 "		33	36	60
1.45 p.m.	Ventilation stopped.			
1.45 "		30	38	60
2.15 "		43	44	58
2.30 "	Oxygen lack. Blood dark. Occasional gasps.			
3.15 "	Reflexes sluggish.	43	44	50
3.40 "	Irregular blood pressure.			50-90
4.00 "	Great O_2 deficiency.	60	54	35
4.10 "	Dog dead.			

Autopsy.—Nothing abnormal found.

From the two preceding experiments it is clear that excessive artificial respiration results in a marked decrease in the CO_2 content and CO_2 capacity of the blood and also in the arterial pressure. In the light of the experiments reported in the two preceding papers of this series it is altogether likely that these results are chiefly due to the excessive ventilation. A possible objection may however be made that the violent movements of excessive artificial respiration are in part responsible for the effects,

particularly upon arterial pressure. In order to test this point therefore we carried out Experiment 5. The point of the experiment was to administer quite as vigorous artificial respiration as in the previous experiments, but with air containing such a percentage of CO_2 as would prevent any reduction in the CO_2 content of the blood.

For this purpose the apparatus shown in Fig. 1 was used with

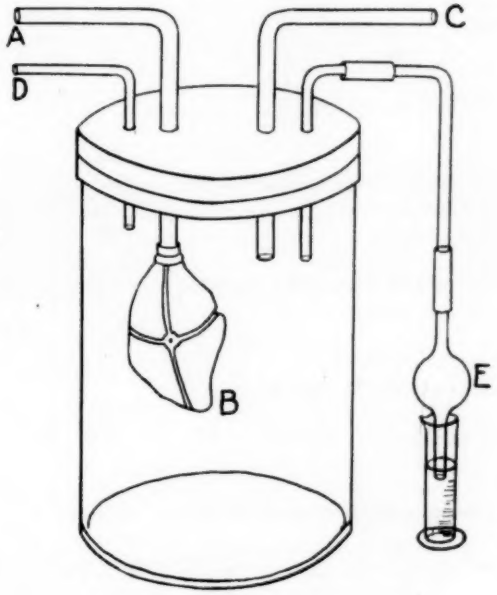

FIG. 2.

the addition of that shown in Fig. 2. The latter consisted of a glass jar of 12 liters capacity in which a rubber bag (B) was suspended on the end of a tube (A) which was fastened to the Y tube of the apparatus of Fig. 1. Tube C was attached to the animal's trachea. Oxygen was passed into the jar as needed, and gas samples were taken, through tube D, while a small escape valve (E, at the right of the figure) allowed a little fresh air to enter and leave the jar at each reversal of the air current.

Experiment 5.—Control experiment. Dog, male, 9 kilos. Excessive artificial respiration with air containing CO_2. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Blood CO_2.		Arterial pressure.
		Content.	Capacity.	
p. m.		*vol. per cent*	*vol. per cent*	*mm.*
2.10	Normal.	47	49	
2.30	Chloratone, 1.8 gm.			
3.00	Ether. Operated.			
3.21	" stopped.	43	46	
3.40				110
3.45	Ventilation started.			
3.50				138
4.15		56	52	
4.40	Contents of rebreathing chamber 7.9 per cent CO_2.			
4.45		58	53	
4.45				142
5.10	Contents of rebreathing chamber 6.7 per cent CO_2.			
5.15		56	54	126
5.22	Ventilation stopped.			
5.45	Respirations, 32 per min.			118
5.55		38	44	90
6.15	Respirations, 37 per min.			75
6.30	" 32 " "			80

Reflexes normal, animal in good condition. Killed.

From this experiment it appears that the movements of excessive artificial respiration so far from causing a fall of arterial pressure in themselves tend to cause (or at least are compatible with) a rise, due either to the pumping action upon the thoracic and abdominal blood vessels or to the effects of high CO_2. The rather low arterial pressure after termination of the period of excessive ventilation may fairly be assigned to the chloratone.

CONCLUSIONS.

Excessive pulmonary ventilation by means of artificial respiration induces not only a lowering of the CO_2 content but also of the CO_2 capacity of the blood. Arterial pressure also falls and death

from circulatory failure may follow[2]. If the artificial respiration, instead of being made with fresh air, is carried on by continual reinjection mainly of expired air, so that the CO_2 content of the blood is not reduced, the CO_2 capacity and arterial pressure do not fall and the other ill effects also fail to appear.

[2] Ewald, A., *Arch. ges. Physiol.*, 1873, vii, 580. Henderson, *Am. J. Physiol.*, 1908, xxi, 126.

RESPIRATORY REGULATION OF THE CO_2 CAPACITY OF THE BLOOD.

IV. THE SEQUENCE OF TRAUMA, EXCESSIVE BREATHING, REDUCED CO_2 CAPACITY, AND SHOCK.

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The term "shock" has come to be applied not only to the most diverse sorts of functional depression following trauma, but also to nearly any experimental method (no matter how artificial) for causing circulatory failure.

The common procedure for the production of shock experimentally in dogs is to expose and manipulate the abdominal viscera under morphine anesthesia. The bearing of such radical experiments on shock in man can be accepted only with considerable reserve. This is important in considering experimental work on shock because of the fact that, as Mann[1] has pointed out, with the exception of certain experiments perform ed some years ago in this laboratory, the procedure of exposure and manipulation of the abdominal viscera has been almost the sole method by which "shock" has been induced experimentally.

In some experiments, of which a preliminary notice was published some months ago by Prince and ourselves,[2] it was found that after irritation of the abdominal viscera a marked reduction of the CO_2 capacity of the blood occurred. In these experiments we intentionally induced excessive (natural) breathing.

Recently Cannon[3] has reported that in wounded soldiers in shock the "alkaline reserve" or CO_2 capacity of the blood is

[1] Mann, F. C., *Bull. Johns Hopkins Hosp.*, 1914, xxv, 205.

[2] Henderson, Y., Prince, A. L., and Haggard, H. W., *J. Am. Med. Assn.*, 1917, lxix, 965.

[3] Cannon, W. B., Reports to Committee on Physiology, National Research Council, *Am. J. Physiol.*, in press.

much reduced, that a further reduction is induced by anesthesia and operation, and that administration of alkali intravenously is beneficial.

In view of these facts, particularly Cannon's observations, it seemed to us important to determine whether trauma *per se* apart from excessive pulmonary ventilation and reduction in the body's content of CO_2 would cause a reduction in the CO_2 capacity of the blood. As Protocols 1 and 2 show, the answer is clearly in the negative. In general the analytical methods employed were similar to those used in the previous papers of this series.

Experiment 1.—Dog, female, 9 kilos. General intestinal trauma. The animal breathed air containing a sufficient percentage of CO_2 to prevent loss due to hyperpnea.

Time.	Condition.	CO_2 breathed.	Blood CO_2.		Arterial pressure.
			Content.	Capacity.	
p.m.		*per cent*	*vol. per cent*	*vol. per cent*	*mm.*
3.20	Normal.		48	49	
3.30	Morphine, 0.18 gm.				
4.30			57	54	122
5.00	CO_2 started.	7.3			130
5.10	Intestinal trauma started.				122
5.40	Intestines very congested.		54	51	100
6.10	" engorged.		54	51	
6.13	Accidental hemorrhage of about 100 cc.				
6.40	Manipulation stopped.	7.6	52	50	78
7.10	Heart stopped. Respiration continued for a minute or two.				

Death in this case was probably due to the large amount of blood which stagnated in the traumatized viscera and to loss of heat. Such a death may be assigned to shock, but it is a shock of a different type from that induced by less trauma when the CO_2 of the body is greatly reduced.

In the following experiments we have employed a method of inducing shock which avoids the objections above pointed out to general visceral trauma. An incision under morphine anesthesia was made in the midline of the abdomen over the stomach barely large enough to admit the hand. The hand carefully wrapped with cotton was then introduced up to the wrist, and the space

around the wrist was packed with cotton to prevent loss of heat or the escape of a loop of intestine. The clenched hand was moved rapidly 50 or 60 times a minute so as to bring the knuckles with a downward and forward motion upon the duodenal end of the stomach. The result was vigorous respiration on the part of the subject. Several times there was vomiting. The animal was kept upon a warm plate and there was no fall of rectal temperature. Any consciousness of pain was prevented by the morphine administered.

In Experiment 2 trauma and excessive breathing were produced in this way. The animal was, however, made to breathe

Experiment 2.—Dog, male, 12 kilos. Gastric manipulation, while the animal breathed CO_2 and later without CO_2. Equilibrating air 5.55 per cent CO_2.

Time.	Condition.	Respirations per min.	CO_2 breathed.	Blood CO_2.		Arterial pressure.
				Content.	Capacity.	
p.m.			*per cent*	*vol. per cent*	*vol. per cent*	*mm.*
1.45	Normal.	26		48	50	
2.00	Morphine, 0.24 gm.					
2.15		16				108
2.45		15		67	60	110
2.53	CO_2 started.		6.8			
3.05	Abdominal incision.	19				120
3.15	Gastric manipulation started.	56				120
3.30		54				120
3.45	Vomited.	43	5.8	56	50	130
4.15		44	7.0			128
4.45		37	7.1	53	56	116
4.53	Manipulations stopped.					
5.10	CO_2 stopped.					
5.15		7				
5.30		7	0	41	49	106
6.30		9	0	46	47	106
6.45	Gastric manipulation.	34				108
7.05	Manipulation stopped.	34				
7.15		9		36	36	70
7.30	Dog dead.					44

air containing a sufficient amount of CO_2 to prevent loss. From the protocol it will be seen that after an hour and 38 minutes

under these conditions the CO_2 content and the CO_2 capacity of the blood were still practically normal, and arterial pressure had not suffered any considerable fall. Both the CO_2 administration and the manipulation were then stopped for 2 hours. No very marked change followed. Then the manipulation was repeated but without inhalation of CO_2. In 20 minutes the CO_2 content and capacity had fallen to the critical level and arterial pressure was undergoing a decline which ended 25 minutes later in death.

In Experiment 3 the manipulation of the stomach was carried out in the manner above described for 1 hour. The animal did not breathe CO_2. 23 minutes after the termination of this treatment death followed.

Experiment 3.—Dog, female, 9 kilos. Manipulation of stomach. Equilibrating air 5.4 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.		Arterial pressure.
			Content.	Capacity.	
p.m.			*vol. per cent*	*vol. per cent*	*mm.*
2.00	Normal.	20	48	49	
3.15	Morphine, 0.19 gm.	10			
3.30		11	61	54	126
3.45	Gastric manipulation started.				
4.00		50	43	52	94
4.15			37	49	
4.30		46	28	38	86
4.45	Manipulation stopped. Reflexes absent.				
5.00		36	27	32	58
5.08	Dog dead.				40

In Experiments 4 and 5 the manipulation of the stomach was carried out until a distinct condition of shock as judged by CO_2 capacity, absence of reflexes, and low arterial pressure was attained. The attempt was then made to reverse these conditions and to restore the animal by causing it to inhale CO_2 in the percentages shown in the protocols. In neither case was there any distinct improvement, and death followed. Autopsy showed little engorgement of the intestines and no hemorrhage, but there was a marked dilatation of the stomach.

Experiment 4.—Dog, male, 7½ kilos. Gastric manipulation, followed by an attempt to treat low CO_2 capacity with CO_2. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Respirations per min.	CO_2 in air breathed.	Blood CO_2.		Arterial pressure.
				Content.	Capacity.	
			per cent	*vol. per cent*	*vol. per cent*	*mm*
10.00 a.m.	Normal.	22		46	49	
10.05 "	Morphine, 0.15 gm.					
10.45 "		10		58	56	110
10.55 "	Gastric manipulation started.					
11.00 "		64				155
11.15 "		52				134
11.30 "		58				120
11.45 "		46		43	47	115
12.15 "		54		30	33	75
12.25 "	Manipulation stopped. Moribund, no reflexes.					
12.30 "	Started CO_2.	39	7.6	26	27	35
12.45 "		20	7.6	31	26	32
1.00 p.m.		18	7.6	33	29	25
1.15 "		21	7.6	34	30	30
1.28 "	Heart stopped.					

Experiment 5.—Dog, female, 7 kilos. Similar to Experiment 4. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Respirations per min.	CO_2 in air breathed.	Blood CO_2.		Arterial pressure.
				Content.	Capacity.	
p.m.			*per cent*	*vol. per cent*	*vol. per cent*	*mm.*
2.15	Normal.	21		50	49	
2.30	Morphine, 0.14 gm.					
3.00		12		59	53	100
3.30	Manipulation started.	47				130
3.45		53				80
4.00		60		39	42	
4.15	Reflexes weak.					40
4.20	Manipulation stopped.					
4.25				34	37	40
4.30	CO_2 started.		8.0			
4.45		30	9.1	41	36	50
5.00			9.7			40
5.15			10.3	46	38	55
5.30			11.0	46	35	25
5.43	Heart stopped.					

Evidently beyond the critical point the processes causing lowering of the CO_2 capacity and other ill effects are not reversible. Perhaps at these low levels a true acidosis due to tissue asphyxia resulting from failing circulation sets in, as suggested some years ago in papers on this general subject from this laboratory.[4]

The matter is particularly interesting now in view of the fact that Porter[5] has recently reported beneficial results by causing wounded soldiers in shock to breathe CO_2. In the light of our observations we should expect this treatment to hasten the recovery of those cases whose CO_2 capacity was still above the critical level and who would therefore in all probability have recovered slowly without treatment, but to be only a means of temporarily prolonging life in cases in which the CO_2 capacity had already sunk below the critical level.

In this connection we may mention the fact that some years ago one of us had an opportunity to observe and treat in this way a case of shock. The case was that of a boy who had been run over by a railroad train. One of his feet had been cut off and the other badly mangled. Apparently there had been no considerable hemorrhage. Apparently also there was, in accord with our correspondent quoted in Paper III, at no time agonizing pain. Nevertheless, even when he first came under observation within an hour after the accident the character of the breathing was such as to indicate a distinct tendency toward apnea. In the course of the next few hours during the night there was Cheyne-Stokes breathing. Next morning he was at the point of death at about 9 o'clock, respiration having reached the stage of asphyxial inspiratory gasps. CO_2 gas was administered by holding an inverted glass funnel connected with a tank of CO_2 above his nose. Under such inhalation respiration was restored to a form strikingly like that of normal sleep. There was a marked improvement in the color of the skin and lips and in other vital signs. The treatment was continued for 8 or 9 hours and certainly prolonged life fully to this extent. Nevertheless the circulation gradually failed and death followed.

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[4] Henderson, Y., *Am. J. Physiol.*, 1908, xxi, 126; 1908–09, xxiii, 345; 1909, xxiv, 66; 1909–10, xxv, 310, 385. Henderson, Y., and Scarbrough, M. McR., *ibid.*, 1910, xxvi, 260. Henderson, *ibid.*, 1910–11, xxvii, 152.

[5] Porter, W. T., *Boston Med. and Surg. J.*, 1917, clxxvii, 326.

CONCLUSIONS.

In reports from Cannon on his observations upon shock in wounded soldiers, emphasis has been placed upon the lowering of the alkaline reserve of the blood, upon the fact that under anesthesia and operation a further lowering occurs, and that intravenous injection of alkaline solution has strikingly beneficial effects.

Our experiments show that apart from overventilation of the lungs trauma does not cause a lowering of the CO_2 capacity (alkaline reserve) of the blood, even when by general traumatization and cooling of the abdominal viscera and consequent stagnation of the blood in the injured parts, failure of the circulation and death are induced.

If, on the other hand, no measures are taken to prevent excessive pulmonary ventilation, the overbreathing induced even by localized manipulation of the stomach, while the rest of the viscera are protected, causes lowering of the CO_2 content and CO_2 capacity of the blood, and also of arterial pressure. If the lowering of the CO_2 capacity is pushed beyond the apparently critical level, between 33 and 36 volumes per cent, the disturbance of the vital equilibrium results fatally.

The same duration and degree of localized manipulation administered while the subject is inhaling 6 or 7 per cent of CO_2 does not cause any marked lowering of the CO_2 capacity, arterial pressure, or general vitality.

Below the critical level for the CO_2 capacity, treatment by the administration of 7 to 10 per cent CO_2 in the air breathed does not cause restoration of CO_2 capacity or arterial pressure.

These results are in general harmony with the conception of acapnia as a factor in shock, long advocated in papers from this laboratory.

Further investigations are under way on the therapeutic and prophylactic sides.[6]

The expenses of the investigations reported in this series of papers have been defrayed by the Loomis Medical Research Fund.

[6] Papers dealing with the effects upon the circulation of excess and deficiency of CO_2 will be published shortly in the *Journal of Pharmacology and Experimental Therapeutics.*